

The formulation and refinement of a polymerase chain reaction (PCR) assay for early diagnosis of paediatric HIV infection and genetic analysis of variants involved in vertical transmission of HIV-I

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ABBREVIATIONS

⁰ C	degrees Celsius
³² P	radio-labelled phosphorus
³⁵ S	radio-labelled sulphur
4'- AMDMIP	4'- aminoethyl- 4,5'- dimethylisopsoralen
AIDS	Acquired immunodeficiency syndrome
ARC	AIDS-related complex
ARV	AIDS-associated retroviruses
bp	base pair(s)
BSA	bovine serum albumin
CDC	Centre for Disease Control
cDNA	complementary DNA
CsCl	caesium chloride
dATP	2'- deoxy-adenosine- 5'- triphosphate
dCTP	2'- deoxy-cytidine- 5'- triphosphate
dGTP	2'- deoxy-guanosine- 5'- triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
ds	double strand
dTTP	2'- deoxy-thymidine- 5'- triphosphate
dUTP	2'- deoxy-uridine- 5'- triphosphate
EBV	Epstein Barr Virus
EDTA	ethylene-diamine-tetra-acetic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EtBr	ethidium bromide
FDA	Food and Drug Administration
g	gram(s)
HCl	hydrochloric acid
HIV-I	Human immunodeficiency virus type one
HIV-II	Human immunodeficiency virus type two
hr	hour(s)
HTLV	Human T-cell leukemia virus
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IPTG	isopropyl-B-D-thio-galactoside
IVAP	<i>in vitro</i> antibody production
IVDU	Intravenous drug users
kb	kilobase pair(s)
LAS	lymphadenopathy syndrome
LAV	Lymphadenopathy - associated virus
Li	lithium
LIP	lymphocytic interstitial pneumonitis
LTR	long terminal repeat
M	moles per litre (molar)
μCi	microcurie(s)
Mg	magnesium
μg	microgram(s)
mg	milligram(s)

MgCl ₂	magnesium chloride
min	minute(s)
μl	microlitre(s)
ml	millilitre(s)
mM	millimoles per litre
μM	micromoles per litre
mRNA	messenger RNA
NaOH	sodium hydroxide
NASBA	nucleic acid sequence based amplification
NH ₄ OAC	ammonium acetate
nm	nanometer(s)
OD	optical density
ORF	open reading frame
PBMC	peripheral blood mononuclear cell
PCP	<i>Pneumocystis carinii</i> pneumonia
PCR	polymerase chain reaction
pmol	picomole(s) per litre
psi	pounds per square inch
RNA	ribonucleic acid
Rnase	ribonuclease
rpm	revolutions per minute
RSV	Rous sarcoma virus
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulphate
SSC	sodium chloride/ sodium citrate
TAE	Tris-acetate
Taq	DNA polymerase from <i>Thermus aquaticus</i>
TE	Tris-EDTA
Tm	melting temperature
TMP - SMX	trimethoprim - sulphamethoxazole
Tris	Tris(hydroxymethyl)aminomethane
U	enzyme unit(s)
UCT	University of Cape Town
UNG	uracil - N - glycosylase
USA	United States of America
UV	ultraviolet
V	volt(s)
v/v	volume per volume
w/v	weight per volume
WB	western blot
WHO	World Health Organization
X-gal	5-bromo -4-chloro -3-indolyl -3-D-galactopyranoside

Abstract

Paediatric human immunodeficiency virus (HIV) infection has become a major socio-economic health problem in recent years as the number of HIV-I infected children steadily increases. The majority of these infants are infected through mother-to-child transmission, with the frequency of vertical transmission varying between 12,9% and 65%. In order to implement appropriate management and possible treatment of these infected neonates, it is essential to have reliable laboratory tests for the early diagnosis of an HIV infection. At the time that this study was initiated, the diagnosis of HIV-I infection in the Groote Schuur Hospital Virology Laboratory depended almost exclusively on serological assays. Such assays are of limited value for infants under 18 months of age, as maternal IgG antibody to HIV-I is transferred via the placenta and may persist in the baby for up to 18 months. Available IgG antibody tests do not distinguish reliably between passively acquired maternal antibody and that produced by the infant itself. A valuable method of establishing the presence of true infection is provided by the polymerase chain reaction (PCR) technique which allows the identification, and subsequent exponential amplification of low levels of specific viral nucleic acid using specific oligonucleotide primers.

A major aim of this study was to develop and instigate a (PCR) assay for the early diagnosis of HIV infection in infected infants. This was successfully achieved by the adaptation and optimization of an existing standard PCR protocol to suit the specific needs of a routine diagnostic service. Preliminary requirements involved the selection of primers and probes and establishing optimal parameters for: ionic strength, *Taq* DNA polymerase concentration, primer concentration, deoxynucleotide triphosphate concentration, and hybridization conditions for most efficient functioning of

the test. The devised method entailed the extraction of proviral DNA from peripheral blood mononuclear cells, amplification of HIV-I specific sequences by PCR, and identification by Southern blot hybridization with digoxigenin (DIG)-labelled probes.

Thereafter the efficacy of the assay was tested on 45 infants (under 15 months of age) all born to seropositive mothers and therefore at risk for HIV infection. Forty-two of these infants had antibodies to HIV-I and the remaining 3 were seronegative. The latter 3 also tested negative for HIV proviral DNA when PCR was performed, using at least 2 different HIV-I primer pairs and their respective DIG-labelled probes. However, 27 (64%) of the 42 seropositive infants were also HIV-PCR positive and the remaining 15 (36%) seropositive infants were negative for HIV proviral DNA. Positive PCR tests correlated well with clinical data indicative of active HIV-I infection for the majority of infants in the neonatal period, although it could not provide proof of infection in newborn babies (less than 1 week of age).

The development of an in-house PCR protocol specific for HIV-I has not only provided a valuable diagnostic assay for neonatal infection, but has also given insight into the parameters required for high sensitivity and the stringent precautionary measures that need to be applied to avoid contamination problems.

The second part of this study was devoted to DNA sequence analysis of cloned HIV isolates from an infected mother and her 3 month old infant. Nucleotide sequence variation between isolates of HIV-I has been well documented. Examination of the third variable region (particularly the V3-loop) in the *env* gene of HIV-I of our mother-infant pair confirmed this variation and provided the first genetic epidemiological data of this nature in the local community.

Proviral DNA from both mother and baby was amplified using V3-specific degenerate primers and cloned. Clones containing the insert DNA were

identified by colony-blot hybridization. Their nucleotide and amino acid sequences were analyzed by using various computer programs. The degree of similarity between variants from the mother and infant in this study differed to a large extent from previous studies. The virus population harboured by the mother displayed highly homogeneous V3 sequences (1,04% variation) compared to the isolates from her 3 month old infant, which showed a higher degree (1,8%) of heterogeneity. Phylogenetic analysis of the different isolates from mother and infant demonstrated that an HIV-I subtype C virus was the infectious agent. This classification was confirmed by the characteristic amino-acid sequence of the tetrapeptide motif of the V3 loop present in the isolates from both mother and infant as well as the absence of a potential N-linked glycosylation site proximal to the first cysteine of the V3 loop, which is characteristic of subtype C viruses.

Based on the amino acids present at positions 306 and 320 of the V3 loop, it could also be concluded that isolates from both the mother and her baby were consistent with the non-syncytium inducing (NSI) phenotype of HIV-I, thus indicating that, contrary to popular belief, NSI variants can be responsible for initiating infection.

Data obtained from these genetic investigations of variants involved in vertical transmission of HIV-I can form a useful basis for future comparative studies.

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Chapter 1

Overview of HIV-I

1.1 Historical Aspects

As the human immunodeficiency virus (HIV-I) continues to infect millions of people throughout the world, understanding the role of this retrovirus as the etiological agent of acquired immunodeficiency syndrome (AIDS) requires an historical detour through the puzzling connection between retroviruses and animal cancers. The first oncovirus was isolated in 1911 when Peyton Rous reported that tumours in chickens could be caused by a virus readily transmissible by filtered extracts (Rous 1911). This virus was later known as the Rous sarcoma virus (RSV). Since then, innumerable retroviruses have been isolated from many groups of vertebrates.

In the early 1960's, a feline cancer-inducing virus was discovered, now called the feline leukaemia virus (Jarret *et al.*, 1964). This virus proved to be important in understanding the biology of retroviruses for two reasons; firstly, it induces an immunodeficiency in cats similar to that later observed in AIDS patients, and secondly, because feline leukaemia virus is transmitted among cats in a household setting, it provided a valuable model for epidemiological analysis of retrovirus infection. Until the late 1960's, there was still considerable scepticism that a virus could mediate the transmission of cancer. Because cancer appeared to be caused by a genetic alteration, it was difficult to conceive how ribonucleic acid (RNA) contained in retroviruses could interact with the deoxyribonucleic acid (DNA) of the host cell to produce oncogenic changes. The discovery of reverse transcriptase suggested a mechanism for the induction of

permanent genetic change (Abrell and Gallo, 1973). This enzyme uses RNA as a template and reverses the conventional flow of genetic information by synthesizing a copy of complementary DNA (cDNA) that ultimately integrates into the genome of the host cells. This DNA, called provirus, serves as an intermediate stage in the replicative cycle of the virus.

The first indication that AIDS could be caused by a retrovirus came in 1983, when Barre-Sinoussi at the Pasteur Institute recovered a reverse transcriptase-containing virus from the lymph node of a man with persistent lymphadenopathy syndrome (LAS) (Barre-Sinoussi *et al.*, 1983). The characteristics described for the retroviruses recovered by the Pasteur Institute group included some that were also reported for the human T-cell leukaemia virus (HTLV) (Blattner *et al.*, 1983). Thus, many investigators decided initially that the lymph node isolate was a member of this already recognized human retrovirus group. The concurrent publication of a paper by Gallo (Gallo *et al.*, 1983) in the same issue of *Science*, reporting the isolation of HTLV from individuals with AIDS, supported this conclusion. However, HTLV as the etiological agent of AIDS seemed unlikely since it was known to be strongly cell-associated and to display poor replication in culture, yet AIDS had reportedly been transmitted to haemophiliacs (Meissner *et al.*, 1992) by cell-free plasma products such as factor VIII. The characteristic loss of CD4⁺ lymphocytes in AIDS patients (Gottlieb *et al.*, 1981) could also not be explained by an HTLV infection, since HTLV does not lyse lymphocytes, in fact, it often immortalizes them into continuous growth (Miyoshi *et al.*, 1981).

Further studies in 1983 by Montagnier and co-workers clarified these questions in relation to the LAS agent (Montagnier *et al.*, 1984). Their results indicated that this human retrovirus, later called lymphadenopathy-associated virus (LAV), although similar to HTLV in its ability to infect

CD4⁺ lymphocytes, had quite a distinct property in that it grew to substantial titre in CD4⁺ cells and killed these targets instead of maintaining them in culture as does HTLV (Montagnier *et al.*, 1984). These observations on LAV provided important evidence to support the potential etiological role of a retrovirus in AIDS. Thus, 3 years after the first description of the disease in 1981, it was shown that AIDS was caused by a retrovirus.

Several other laboratories were also searching for the agent responsible for the immune deficiency syndrome, and in early 1984 Gallo and associates reported the characterization of another human retrovirus, distinct from HTLV, which they called HTLV-III (Gallo *et al.*, 1984). It was isolated from the peripheral blood mononuclear cells (PBMC) of adult and paediatric AIDS patients. At the same time Levy and his co-workers reported the identification of retroviruses which they named the AIDS-associated retroviruses (ARV) (Levy *et al.*, 1984). These viruses were recovered from AIDS patients from different known risk groups, as well as from other symptomatic and healthy people. Finding ARV in asymptomatic individuals was the first indication of a carrier state for the AIDS virus. This retrovirus grew substantially in PBMC, killed CD4⁺ lymphocytes and did not immortalize them. Thus, the three newly identified retroviruses had similar characteristics. Most importantly, infection by these viruses was not limited to AIDS patients. They were also recovered from individuals with other clinical conditions, including lymphadenopathy.

Within a short period, the three prototype viruses were recognized as members of the same group of retroviruses. Their proteins were all distinct from those of HTLV and their genomes showed only remote similarities to the genome of that agent. Thus, the suspected cross-reactivity of HTLV-III with HTLV was proved to be incorrect. For all these

reasons, in 1986, the International Committee on Taxonomy of Viruses recommended that the AIDS virus be given a separate name, the human immunodeficiency virus (HIV) (Coffin *et al.*, 1986). Soon after the discovery of HIV type one (HIV-I), a separate subtype, HIV type two (HIV-II) was identified in western Africa (Clavel *et al.*, 1986). Both HIV subtypes can lead to AIDS, although the pathogenic course with HIV-II might be longer.

1.2 Classification

The retroviruses belong to the family Retroviridae, which is taxonomically divided into three subfamilies, based primarily on pathogenicity rather than on genome relationships. The 3 subfamilies comprise the *Oncovirinae*, which includes those retroviruses that cause tumours in the host, or transformation in tissue culture (HTLV-I and -II belong to this subfamily); the *Lentivirinae*, which includes a number of viruses that cause slow virus diseases with progressive degeneration of the central nervous system (HIV-I and HIV-II are included in this subfamily) and the *Spumavirinae* including the foamy viruses which may result in persistent infections and vacuolar pathological findings in tissue culture, accounting for their name (Coffin, 1990; Dodd, 1994; Dalglish and Weiss, 1990).

The subfamily *Lentivirinae*, to which both HIV-I and HIV-II belong, includes a large number of different viruses that infect a diverse group of animal species (Table 1). The genomes of these viruses are characterized by a complex combination of genes encoding multiple structural proteins and non-structural regulatory gene products.

Table 1 Lentivirinae

Group	Virus	Host	Disease
Lentiviruses	Human immunodeficiency virus (HIV type I and II)	Human	Cause of AIDS
	Simian immunodeficiency virus (SIV)	Primate	AIDS-like disease in certain monkeys
	Feline immunodeficiency virus (FIV)	Cat	Immunodeficiency
	Bovine immunodeficiency virus (BIV)	Cattle	Lymphadenopathy Lymphocytosis
	Visna virus	Sheep	Neurological and lung disease
	Equine infectious anemia virus (EIAV)	Horse	Hemolytic anaemia
	Caprine arthritis-encephalitis virus (CAEV)	Goat	Hemolytic anaemia Encephalopathy

A method of classification has been proposed whereby the retroviruses are classified according to their genomic organization and gene regulation. This new system divides the retroviruses into 2 main groups, the Classic retroviruses, which will include the family *Oncovirinae*, and the Complex retroviruses, which will include all retroviruses without oncogenes but with extra regulatory genes (Cullen, 1991).

1.3 Virion Structure

In common with the other retroviruses, HIV-I is a single positive-stranded RNA virus consisting of spherical particles slightly more than 100 nanometers (nm) in diameter. By electronmicroscopy, the virions have the characteristics of a lentivirus, with a dense, cone-shaped protein core. This encases two identical genomic RNA molecules and viral

enzymes required for early steps of replication. The RNA molecules resemble eukaryotic messenger RNA (mRNA) because they contain a cap structure at the 5' end and a poly-adenylated sequence at the 3' end (Meissner and Coffin, 1992; Wong-Staal, 1990). The major structural proteins encasing the RNA are the p24 capsid protein and p17 matrix protein.

The outermost layer of the virion (Fig. 1) consists of a lipid bilayer derived from the outer membrane of the host cell. This viral envelope contains numerous proteins of both viral and host-cell origin. Studding the outer membrane of the virus are morphological projections composed of the envelope glycoproteins, gp120 and gp41. Each stud comprises four molecules of gp120 non-covalently attached to four molecules of gp41 which are embedded within the membrane (Pinter *et al.*, 1989). These envelope proteins play a crucial role when HIV binds to and enters target cells (see chapter 1.4).

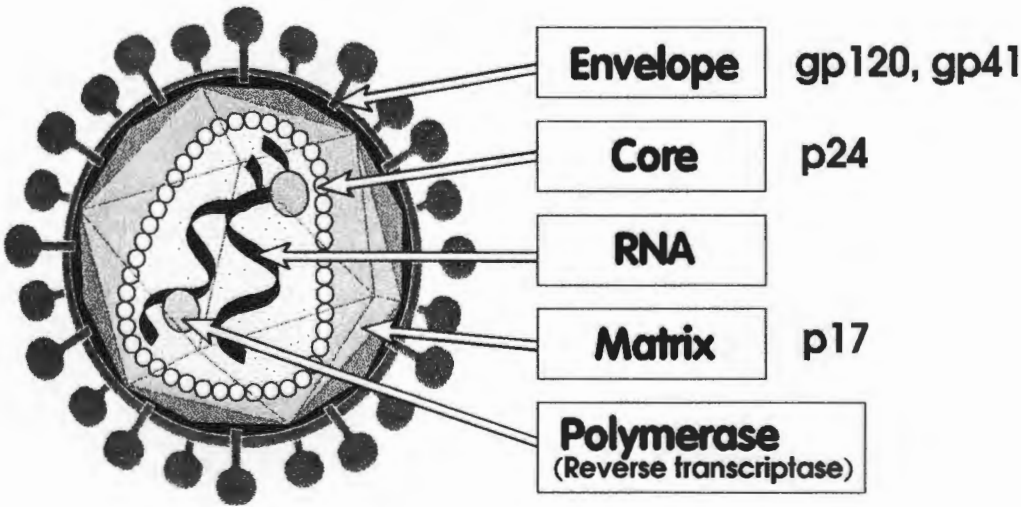


Fig. 1 A schematic drawing of the structure of the HIV-I virion.

1.4 Genomic Organization

In general, the genomic size of HIV is about 9,8 kilobases (kb) with overlapping open reading frames (ORF) coding for several viral proteins. The HIV genome is a full-length viral mRNA which contains two long terminal repeat (LTR) elements, one at each end, along with three structural genes that are essential for virus replication, namely the *gag*, *pol* and *env* genes (Fig. 2) that code for the core, polymerase and envelope proteins respectively.

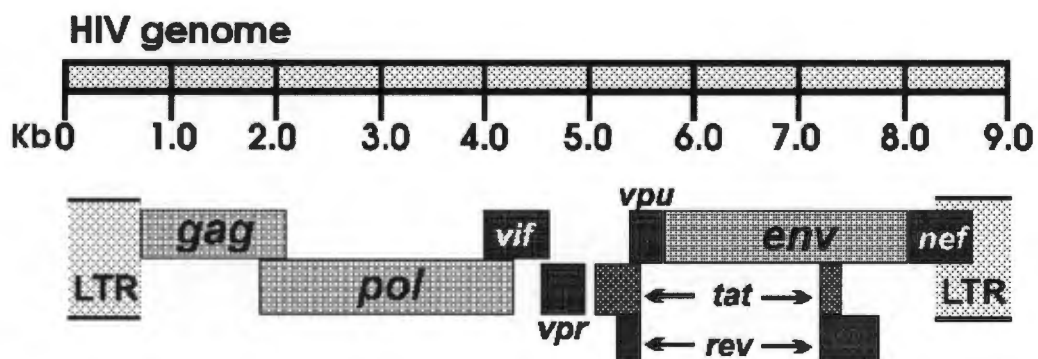


Fig. 2 Genomic structure of HIV-I provirus (Adapted from Greene, 1993).

The *gag* proteins are made as a single polypeptide chain, p55 which is cleaved late in virus maturation by a viral protease, giving rise to smaller proteins:- p24 and p17 which form the virion core; p15 which is RNA associated, and p6 and p2 which are found inside the core (Greene, 1993).

The *pol* precursor protein is post-translationally cleaved into products consisting of the reverse transcriptase, the protease and the integrase proteins. These enzymes occur in the precursor protein in the following order (amino-terminus to carboxy-terminus):- NH₂, protease, reverse

transcriptase, integrase, C00H (Wong-Staal, 1990; Meissner and Coffin, 1992).

The envelope proteins, gp120 and gp41 are made as a single large polypeptide, gp160. This envelope protein is transported to the cell surface where it is cleaved by cellular proteases. The amino-terminal portion of the protein is located entirely exterior to the cell membrane (gp120) and is non-covalently associated with the carboxy-terminus (gp41) that is anchored to the viral membrane. The association of the envelope proteins with the core occurs as the virus buds from the cell surface (Haseltine, 1990; Wong-Staal, 1990; Gallaher *et al.*, 1995).

In addition to these structural genetic elements, HIV-I contains at least six accessory genes: *tat*, *rev*, *nef*, *vif*, *vpr* and *vpu*. Three of these are viral regulatory proteins: *tat*, *rev* and *nef*, with *tat* being a transactivating protein that, along with certain cellular proteins, interacts with an RNA loop structure formed in the 3' portion of the viral LTR called TAR (*tat* responsive region), and upregulates HIV replication. The viral regulatory protein, *rev* (regulator of viral protein expression), interacts with a *cis*-acting RNA loop structure called the *rev* responsive element, located in the viral envelope mRNA. This interaction involves cellular proteins and multimers of the *rev* protein and permits unspliced mRNA to enter the cytoplasm from the nucleus and give rise to full-length viral proteins needed for progeny production. Thus, *tat* and *rev* are RNA-binding proteins that interact with cellular factors for optimal activity. The third protein, *nef* (negative factor) appears to have a variety of potential functions initially thought to include down-regulations of viral expression (Greene, 1991; Wong-Staal, 1990), although more recent experiments have cast doubt on this. It appears that the *nef* protein may somehow modify the cell to provide a more appropriate environment for viral

maturation, and that production of *nef* protein is required for the development of AIDS (Greene, 1993).

The other three genetic elements *vif*, *vpr* and *vpu* are accessory proteins of which *vif* is presumably involved in the infectivity of free virus; *vpr* protein is found in the virion but the function is unknown and *vpu* protein is found in the cell membrane and may be required for optimal assembly/packaging of virions and the promotion of extracellular release of viral particles. Thus, all three seem to be significant in HIV pathogenesis (Myers *et al.*, 1992; Wong-Staal, 1990; Greene, 1993).

1.5 Life Cycle of the Virus

Once an HIV virion has entered a cell, a complex sequence of events follows which, if completed, leads to the budding of new virus particles from the infected cells. The replication cycle of HIV (Fig. 3) includes the following steps: (1) Attachment and penetration, (2) Reverse transcription and integration, (3) Transcription and translation and (4) Assembly and release of progeny virions.

1.5.1 Attachment and penetration

Virus infection requires interaction of the viral envelope glycoprotein complex with the CD4 molecule, which serves as a high-affinity receptor. The CD4 molecule is a surface glycoprotein whose physiological function is to bind class II major histocompatibility (MHC) molecules on the surface of antigen-presenting cells. It is present on a variety of hematopoietic cells, including T lymphocytes, macrophages, and dendritic cells. Entry occurs via fusion of the viral and cellular membranes. This is triggered by a conformational change within the envelope glycoprotein complex that

occurs as a result of CD4 binding (Putney, 1992). A highly hydrophobic region located at the N-terminus of the membrane-bound gp41 molecule, called the fusion peptide, is believed to be “activated” and to mediate fusion of viral and cellular membranes. The subsequent internalization and uncoating processes are still poorly understood (Dalglish *et al.*, 1984; Klatzmann *et al.*, 1984).

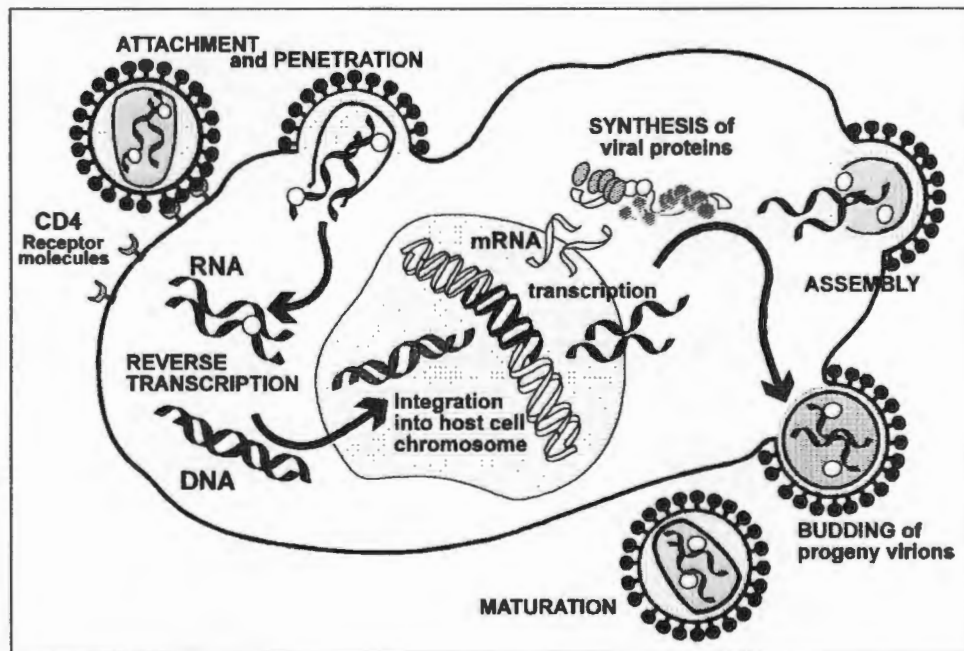


Fig. 3 Diagrammatic representation of the life cycle of HIV-I.

Recently the possibility that infection with HIV might also start with invasion of intact epithelia has been considered. This possibility was ignored in the past, because epithelial cells do not express CD4, the principal cell surface receptor for HIV. Several reports have, however, concluded that HIV can infect CD⁺ cells, including epithelia (Adachi *et al.*, 1987; Zachar *et al.*, 1991). Evidence has also been presented that *in situ* hybridization can be used to detect HIV-infected epithelial cells in the gut of an AIDS patient (Nelson *et al.*, 1988). Several theories have been put forward to explain how HIV particles could infect intact epithelia and

these include: 1) infection mediated by receptors other than CD4; 2) uptake by non-specific phagocytosis, followed by escape from the phagocytic pathway; and 3) uptake by specialized cells of the immune system that are scattered among epithelial cells (Phillips *et al.*, 1994).

Recent reports have also suggested that gp120 can bind to galactocerebroside (galactosyl-1-ceramide: GalC) and its derivatives. This suggests that GalC is a receptor for HIV infection in neural cells. GalC and its derivatives are important components of myelin and are expressed on the surface of oligodendrocytes in humans and rats. The binding of gp120 to GalC could therefore disrupt myelin formation (Kimura-Kuroda *et al.*, 1994).

After the virion has attached to the cell-surface receptor (a process sometimes called "docking") the cell membrane and the virus envelope fuse. As a result, the viral genomic RNA and accompanying proteins are released into the cell's cytoplasm (Fox, 1991).

1.5.2 Reverse transcription and integration

Once the viral genome has entered the cell as a ribonucleocapsid, the viral RNA, still associated with core proteins, it undergoes reverse transcription (Bukrinsky *et al.*, 1992) using its own RNA-dependent DNA polymerase and RNase H enzymes. The reverse transcriptase enzyme traverses the chain of viral RNA and synthesizes a complementary DNA molecule (cDNA) corresponding to the viral RNA genome. A second DNA strand is synthesized, resulting in a double-stranded (ds) DNA molecule (Lori *et al.*, 1992). During this process, portions of ends of the genomic RNA are copied twice, leading to a structure at each end of the DNA

called the LTR (the LTRs serve as initiation sites for the translation of enzymes used later in the life cycle). The reverse transcription process is not particularly efficient and can result in a mutation approximately every two-thousand bases. The newly synthesized dsDNA is in circular form (Fox, 1991; Greene, 1991).

These ds DNA copies of the viral RNA then migrate to the nucleus, where, as circular but non-covalently bound molecules, they are able to integrate into the cell chromosome. In the integrated state, viral genetic material is called the provirus. Integration appears to occur at random sites, but is thought to be an essential process for the production of progeny virions. There is speculation that HIV-I can be produced without integration (Greene, 1991; Fox, 1991) since this phenomenon has been described for the visna lentivirus, although it has not been confirmed (Harris, 1984). Integration is catalysed by integrase, a virus-coded enzyme, and involves joining the ends of each LTR to cut ends of cellular DNA. When unintegrated, the viral DNA is unstable in the cells and decays after a few days. Recent observations on the early events in viral infection have revealed that HIV undergoes integration and replication in permissive activated T cells, within 24 hours (Kim *et al.*, 1990). In macrophages, the process is similar but progeny production appears to occur after 48 hours (Munis *et al.*, 1992).

1.5.3 Transcription and translation

After the provirus has become established in the host cell's chromosome as a new set of genes, two phases of infection take place. The first represents the **latent phase** of the infection in that the new genes for HIV are inactive, with no viral RNA or proteins being expressed. A reservoir of latently infected cells in the host can be a common phenomenon in HIV

infection (Schnittman *et al.*, 1989). Several hypotheses have been presented to explain the mechanism of cellular latency with HIV. This latency could be caused by methylation of certain portions of the integrated viral LTR needed for the induction of the replicative process, or by methylation of extrachromosomal viral DNA sequences (Bednarik *et al.*, 1987). It could also result from an inactivation of the *tat* or *rev* gene (Drysdale and Pavlakis, 1991). The integrated virus can be activated by a variety of stimuli such as antigenic/cytokine stimulation or co-infection with other viruses. The outcome is a high level of transactivation, resulting in a burst of virus production and rapid death of the cell. As previously mentioned, there are at least two HIV genes (*tat* and *rev*) that function as transactivating factors, which causes an increase in the expression of viral RNA and proteins. During this second **productive phase**, viral DNA is transcribed into messenger RNA by host cell RNA polymerase II. This process cannot start until the polymerase is activated by various molecular switches located in the LTRs.

Two distinct phases of transcription follow. In the early phase, which lasts roughly 24 hours, RNA transcripts produced in the cell's nucleus are snipped into multiple copies of shorter sequences by cellular splicing enzymes. These early-phase short transcripts encode only the viral regulatory proteins, the structural genes are not transcribed. In the late phase, two new size classes of RNA - long (unspliced) transcripts and medium-length (singly spliced) transcripts - move out of the nucleus and into the cytoplasm. These longer transcripts encode HIV structural and enzymatic proteins.

1.5.4 Assembly and release of progeny virions

Once the long and medium-length transcripts reach the cytoplasm, the components for new virions are synthesized and transported to the site of assembly at the cell membrane. Assembly of progeny virions occurs at the cell surface. *Gag* and *gag/pol* precursor molecules are transported to the cell membrane via a cytoplasmic pathway, whereas envelope glycoproteins use the secretory pathway (Bolognesi *et al.*, 1978). Virus assembly is orchestrated by the *gag* polyprotein, which interacts with the *gag/pol* precursor, the accessory proteins *vpx* and *vpr*, and the genomic RNA, thereby facilitating their incorporation into virions. Selective incorporation of viral envelope glycoproteins is also believed to involve *gag* elements although these mechanisms are less well understood (Yu *et al.*, 1992). After release from the cell surface, *gag* and *gag/pol* polyproteins are cleaved within the virus particle by viral protease. This step, which has a morphological correlate in the condensation of the particle core, is absolutely required for viral infectivity (Kohl *et al.*, 1988).

1.6 Genome Variability

A characteristic of HIV is the very high degree of variability exhibited by some portions of the genome, particularly in selected segments of the *env* gene. This results in considerable antigenic heterogeneity between different isolates of the virus. Much has been learned about the molecular characteristics, mechanisms of gene expression and genomic complexity since the discovery of HIV-I and HIV-II. Isolates of both virus types have been characterized, their genomes cloned and their nucleotide sequences determined (Myers, 1992) and it has been shown that genetic

variation is a hallmark of these viruses. This variation could have an impact on many aspects of the biology of HIV, including (a) tissue and target cell specificity, (b) clinical spectrum of pathogenesis and (c) geographic and temporal distribution of the virus, and it also has bearing on the prospects of developing a broadly cross-reactive vaccine. This genotype diversity accumulates at a rate of approximately one nucleotide substitution per genome per replication cycle and is responsible for the rapid generation of drug-resistant and immunological escape mutants in infected individuals (Larder *et al.*, 1989; Phillips *et al.*, 1991). No two HIV strains are alike, and even within a single individual, HIV is present as quasi-species (a swarm of microvariants that are highly related, yet genetically distinct from each other) (Martins *et al.*, 1992; Milich, 1993; Howell *et al.*, 1991).

The first molecular description of HIV heterogeneity was based on Southern blot hybridization analyses of independent virus isolates that showed distinguishable restriction enzyme patterns (Shaw *et al.*, 1984). Heteroduplex analysis of two divergent HIV-I viruses, annealing under conditions of increasing stringency, revealed substitution loops that first appeared in the envelope region of the heteroduplexed molecules (Hahn *et al.*, 1985).

Subsequent nucleotide sequence comparisons confirmed that sequence differences among different viral genomes were not evenly distributed throughout. HIV genes that code for internal viral proteins (*gag* & *pol*) showed relative stability from one isolate to the next, but the *env* gene displayed frequent mutations that lead to variation of its glycoprotein products. Not only is the degree of divergence different among these *env* genes, but the type of nucleotide and amino acid changes are very different. Nucleotide sequence changes in the *gag* and *pol* gene are due to point mutations, whereas changes in the *env* gene are due to deletions,

insertions and duplications. Furthermore, the majority of changes in *gag* and *pol* are in the third base position of the codon, resulting in silent mutations. In contrast, more than half of the single nucleotide changes in *env* occur in the first or second base positions, resulting in amino acid changes.

Despite the considerable variability of the HIV-I envelope proteins they do contain regions or structural features that are highly conserved. Within gp120 there are several highly conserved regions (C1-C4) that are interspersed with regions of high variability (V1-V5) (Fig. 4). These hypervariable regions permit the virus to present new antigenic configurations to the host. The V3 loop is the third variable region of the *env* polyprotein. It comprises a 35 amino acid stretch of conserved length, linked to two cysteine residues by a disulphide bond, and plays a role in the cell-virion fusion process following receptor-binding. Type-specific antibodies to the V3 loop have been shown to neutralize this process (Putney and McKeating, 1990). Thus, the V3 loop has been a natural target of interest in vaccines and passive immunotherapy and V3 sequences are the most numerous of the HIV gene sequences in the database. To date, HIV-I isolates can be divergent in as much as 13% of their genomes and in 30% of their extracellular envelope proteins (Murphy *et al.*, 1993; Ait-Khaled and Emery, 1993; Cretz *et al.*, 1994; Van den Haesevelde *et al.*, 1994).

Assuming a constant rate of evolution of HIV-I which is comparable to that of other retroviruses, it has been estimated that HIV-I entered the human population about 50 years ago (Wong-Staal, 1990).

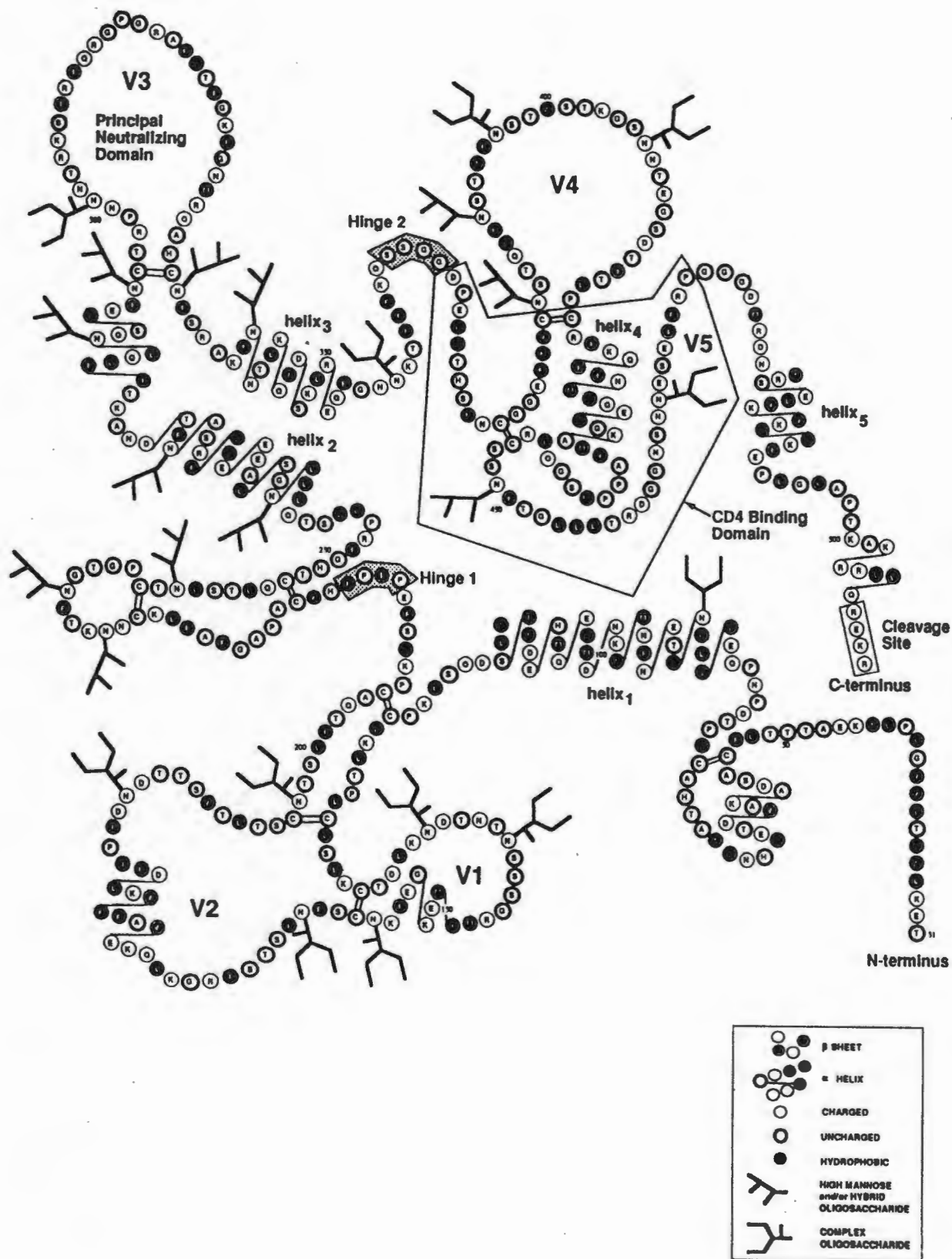


Fig. 4 A computer derived model of the surface glycoprotein, gp120, of HIV-1 with double lines showing the proposed disulfide bonds (Gallagher *et al.*, 1995). The V3 loop is depicted in the upper-left corner.

1.6.1 Subtypes of HIV-I

Myers and his co-workers first attempted to describe HIV subtypes using the *env* sequences. As mentioned before, *env* is the most variable of the larger genes, and it is thus expected that *env* sequences give the most phylogenetic information for closely related viruses (Myers, 1992). When the first isolates from Europe and North America (Ratner *et al.*, 1985; Wain-Hobson *et al.*, 1985) were compared to the first isolates from central Africa, it became apparent that the African isolates were far more diverse at sequence level. At phylogenetic level, all European and North American isolates formed a discrete cluster within the radius of the African viruses. This, along with other epidemiological data, strongly indicated that the common ancestor of HIV-I originated in central Africa, and that one of several lineages later spread to North America (Myers *et al.*, 1988).

Through the impact of viral sequencing of many different isolates from different parts of the world, at least five genetic clusters of HIV-I could be identified by 1992. The North American cluster had been named subtype B. This group now contains isolates from most continents, which probably reflects the radiation of HIV-I to those locations from North America. The African isolates are clustered in a group, termed subtype D. All the viruses so far known to fall within this group came from Central Africa. Viruses now placed in subtype A are from Western and Central Africa. Subtype C includes isolates from South Africa, India and Zambia, while members of subtype E are prevalent in Thailand. The more recently described cluster, termed subtype F, included viruses from Brazil, Romania and Zaire. Subtypes B and D appear to be somewhat more closely related to each other than to the other lineages. This may indicate that subtype B viruses, which initially brought AIDS to prominence in North America were, derived from a virus in Central Africa where the D subtype

is now found (Sharp *et al.*, 1994). HIV-I is currently divided into two major groups, group M and group O.

While Myers continued his study, Louwagie and co-workers (1993) independently examined the diversity among HIV-I isolates from 15 different countries on four continents by using a different genomic region, the *gag* gene. The phylogenetic trees based on the *gag* and *env* sequences were very similar for the majority of isolates but there were some interesting differences e.g. one of the earliest characterized African isolates has an *env* sequence similar to subtype D viruses, but in the *gag* tree it falls within subtype A. Another example concerns more recently characterized viruses from Thailand that fall within subtype E on the basis of their *env* sequences, but cluster with subtype A on the basis of *gag* sequences. It is speculated that this probably reflects recombinations (Sharp *et al.*, 1994). Meanwhile, two additional subtypes have been assigned on the basis of *gag* sequences, namely subtypes G and H, which each include viruses from Zaire and Gabon. This clearly indicates the need for co-ordinates of subtype assignments as well as generally accepted classification criteria.

Recently two HIV-I isolates from Cameroon were sequenced and compared to the different subtypes. Both isolates showed an extraordinarily high level of diversity when compared to the subtypes A - H of group M, and have been termed subtype O (group O) to indicate their outgroup position (Gurtler *et al.*, 1994). It was suggested, based on these findings, that subtype O may represent a second major HIV-I cluster which differs from the first in its evolutionary history, but probably also consists of numerous distinct sequence subtypes. Compared to reference HIV-I strains, the percentage homology of the *gag*, *pol*, and *env* gene products of HIV-I subtype O isolates is in the region of 68%, 74%, and 50% respectively, while >90% homology has been found between O isolates.

Commercial manufacturers have also been advised to modify their HIV test kits to improve HIV-I subtype O sensitivity.

1.6.2 Genomic HIV-I variation in mother and child after transmission

A high degree of genetic variability (see section 1.5) in HIV-I can be found within infected individuals. Variations arise during retroviral replication by error-prone reverse transcription. The complex mixture of variants (quasi-species) in an infected individual may be the result of immunologic pressure for change, alterations in cell tropism or replication efficiency among the variants. Studies of the relationship between virus populations in HIV-I infected mothers and their infants indicate the role of selection in perinatal HIV-I transmission (Wolinski *et al.*, 1992; Mulder-Kampinga *et al.*, 1993; Scarlatti *et al.*, 1993). The virus population observed in the child at birth represents a variant in the mother at the actual time of transmission earlier in pregnancy.

Wolinski and his colleagues (1992) looked at viruses obtained from three mothers and their infants, who ranged in age from two to four months. They sequenced the V3 region containing the immuno-dominant loop and the V4 - V5 regions that encompass a portion of the CD4-binding domain of the viral envelope gene. As expected, the virus transmitted from a mother to her infant was clearly more related to her own virus than to the virus in either of the unrelated infants. The V3 region nucleotide sequences varied between 0,59% and 6,1% for related pairs and 10% - 17,3% for unrelated pairs. An important observation was that although each mother clearly had numerous viral variants in her body, only a subset of these variants were detected in her infant. In all three cases, the virus found in the infants was lacking a site found in most (but not all)

of the mother's viruses where a sugar molecule attaches to a portion of the V3 region. The significance of the missing sugar is still unclear, but the occurrence is so rare that it suggests that some selection is occurring. In addition, although there was clear evidence of viral variants in the infants, the degree of variability in the infant V3 and V4 - V5 was far less than in the mother. It was concluded from this study that only a few of the possible variants were passed on from mother to child.

Mulder-Kampinga and co-workers (1993) examined the genetic relationship between the virus population in an HIV-I infected mother and her infant, and observed a continuous change in sequence populations in the mother. During pregnancy, a nearly complete replacement was detected at position 308 of the V3 loop, from a proline to a histidine amino acid. Replacements of amino acids at position 308 have been shown to influence the antigenic properties of peptides mimicking the central region of the V3 loop (Wolfe *et al.*, 1991). In the child, only a proline was observed in that position during the first weeks of life and remained unchanged until the age of 9 months. This may result in an antigenic distinction between the virus population in the mother and child at time of delivery. The sequence population in the child was highly homogenous (up until the age of 6 weeks). It was characterized by one particular sequence which appeared to represent the variant that had initiated the infection in the child as it was the major variant in the cord blood, although only a minor variant in the maternal sample collected during delivery. This excluded the possibility that viral RNA in the cord blood was due to contamination with maternal blood during delivery and suggests intra-uterine acquired infection. The observed limited variation within the first 6 weeks could mean that the infant had become infected shortly before birth. However, limited variation could also be due to a lower level of replication of HIV-I *in utero* compared to the period after birth, or to a

lower immune pressure in the unborn child and neonate (Borkowsky *et al.*, 1992).

The implication is that some HIV strains are more infectious than others in mother to child transmission. If this proves to be true, then future efforts to prevent perinatal infection can be targetted more accurately.

1.7 Transmission

Epidemiological studies conducted during 1981 and 1982 first indicated that the major routes of transmission of HIV-I were intimate sexual contact and contaminated blood (Jaffe *et al.*, 1983). The syndrome (AIDS) was initially described in homosexual or bisexual men (Gottlieb *et al.*, 1981 and Siegel *et al.*, 1981) and intravenous drug users, but it's occurrence as a result of heterosexual activity was soon recognized as well (Harris *et al.*, 1983). Moreover, it became evident that transfusion recipients and haemophiliacs could contract the illness from blood or blood products, and that mothers could transfer the causative agent to newborn infants (Ammann *et al.*, 1983). These three principal means of transmission - blood, sexual contact and mother-to-child - have not changed. The different groups that are at risk for HIV transmission will be discussed.

1.7.1 Risk Groups

1.7.1.1 Homosexual men

The concept initiated by western observers that AIDS is primarily a disease of homosexual men, is rapidly becoming obsolete. The most

prominent risk factors for acquisition of infection in homosexual men are a high number of sexual partners and participation in sexual practices (mainly receptive anal intercourse) that increases the risk of transmission due to damage of the anorectal mucosa which allows the virus to enter the bloodstream. Direct infection of the epithelial cells of the rectum can also occur (Meissner and Coffin, 1992). In the United States of America (USA), the incidence of new AIDS cases since the early 1980's has reflected changes in the sexual behaviour of gay men, such as decreased numbers of sexual partners and protected anal intercourse. In San Francisco, the seroconversion rate in homosexual men has peaked at about 6000 new HIV infections per year in 1982 or about 10%-15% of the gay male population per year. The rate then declined sharply to 3%-5% by 1984. Since then it has continued to fall, but more slowly, to about 1% per year in 1988 (Bachetti and Moss, 1989). The majority of HIV-infected gay men in San Francisco were infected in the 1979-1983 period. Using the full pattern of seroconversion and incidence data for San Francisco, the average incubation period for clinical AIDS can be estimated to be 9.8 years from infection (Bachetti and Moss, 1989). The same flattening in the incidence of observed AIDS cases in gay men has been reported in New York, with reported AIDS cases peaking in 1987 and declining slightly since then. A similar trend has also occurred in Los Angeles. Thus, in the three major epidemic cities in America, AIDS cases in homosexual men seem to have peaked, and after a period of approximate stability, they can be expected to decline over the next few years.

The decline in the gay male HIV epidemic can be seen as a triumph for health education. For homosexual men questions of prevention are now giving way to questions of early intervention and management of early HIV disease as the central issues in the medical politics of AIDS.

1.7.1.2 Intravenous drug users

As the AIDS epidemic has continued, new AIDS cases in the second major risk group, intravenous drug users (IVDU), has risen, both in the USA and in western Europe. In the USA, IVDU cases rose from 14% of new AIDS cases in 1987 to 20% in 1988 (AIDS update, 1989). AIDS is still believed to be under-reported in IVDUs as some clinical manifestations of HIV infection common in drug users are not regarded as reportable AIDS. It is estimated that AIDS in drug users is under-reported by as much as 50% (Stoneburner *et al.*, 1988). The clinical pattern of AIDS in drug users is different from that in homosexual men, thus clinical experience based on homosexual men may be inappropriate in the case of IVDUs (Des Jarlis *et al.*, 1988). Drug users in the USA are largely impoverished blacks or hispanic, and in Europe they are predominantly unemployed youth. In both cases IVDUs are from social strata which are out of contact with the health care system and unresponsive to health education. Thus the prospects for long-term prevention in this group are less encouraging.

Transmission among drug users occurs through sharing of contaminated needles and syringes that contain a residue of blood (including infected white blood cells). Sero-positivity among drug users correlates with frequency of injection, needle sharing and demographic factors (Meissner and Coffin, 1992).

1.7.1.3 Transmission by blood transfusion

In the years before screening of blood, transfusion recipients and haemophiliacs could be infected by HIV present in blood and blood products such as factors VIII and IX. The screening of blood for anti-HIV antibodies has now reduced the risk of infection by transfusion. Since 1985, HIV transmission by blood transfusion has been exceedingly rare although more than 12 000 individuals may have been infected by this route before that time (Meissner and Coffin, 1992).

The potential risk of infection to transfusion recipients depends on the virus load, which appears to be greatest when an infected individual (as donor) advances to disease. In one study, the chance of infection was reported to be substantially increased if the donor developed AIDS within two to three years after donation. Other studies have also shown that individuals receiving blood from donors who subsequently developed AIDS within 29 months had a greater chance of progressing to disease than those recipients of infected blood from donors who became symptomatic after this period (Ward *et al.*, 1989). In haemophiliacs, the transmission of HIV could be caused only by free virus and was associated with the receipt of many vials of unheated clotting factors thereby increasing the chance that an infectious HIV particle was present in the lyophilized product (Goedert *et al.*, 1989). Nevertheless, evidence of viral nucleic acid in old clotting-factor preparations has been substantiated by subsequent analysis using the polymerase chain reaction (PCR) (Semple *et al.*, 1991). Attempts to isolate infectious HIV from these preparations have thus far been unsuccessful.

At the present time, factor VIII concentrates are unlikely to contain HIV because plasma products are treated with heat or chemicals to inactivate contaminating viruses. Factors VIII and IX are heated to 60°C either before or after lyophilization (Levy *et al.*, 1985).

1.7.1.4 Heterosexual transmission

Studies indicate that spread by heterosexual activity is now responsible for the large majority of infections worldwide (Stoneburner *et al.*, 1990). Seventeen percent (17%) of individuals who acquire HIV by heterosexual contacts are male and 83% are female (Meissner and Coffin, 1992). Transmission occurs more readily from infected males to female contacts than vice versa, but it does take place in both directions. However, this

apparent bias should be viewed with caution since very few female-to-male studies have been published mainly because most index cases in the Western epidemic are male. In a review of 16 cohort studies (Haverkos and Baltjes, 1992), male-to-female and female-to-male transmission rates were compared. Transmission rates varied widely among the studies, with higher rates generally observed in couples recruited in Africa or Haiti (female-to-male transmission 58%; male-to-female transmission 53%) than couples recruited in Europe or the USA (female-to-male transmission 15%; male-to-female transmission 28%).

Heterosexual transmission of HIV is more common in certain countries. In Western countries, as a whole, heterosexual transmission is relatively rare in the general population because transmission probabilities are low, ulcerative sexually transmitted diseases (STDs) are rare and the average number of sexual partners per year is close to one (Skegg, 1989). Regardless of differences in rates of transmission in the various groups, the prevention of major heterosexual epidemics must focus on all young sexually active individuals.

The risk of transmission through oral sex during homosexual and heterosexual contacts is another controversial field. Several case reports suggest that transmission can occur via this route. During the follow-up (Detels *et al.*, 1989) of initially seronegative homosexual men in a highly infected USA population, two seroconversions occurred among men practising fellatio but not anal sex, leading to a seroconversion rate of 0,6 per 1000 persons followed for 1 year.

Although sex with condoms should not be considered as "safe sex", it is definitely considered as "safer sex". During a prospective study of heterosexual couples in which one partner was HIV-negative, no seroconversion was found to occur among 100 partners who used

condoms during each episode of vaginal or anal intercourse, while 10 seroconversions occurred among 104 partners who did not systematically use condoms (De Vincenzi and Ancelle-Park, 1991).

1.7.1.5 Vertical transmission

Infection of children with HIV-I has been recognized since 1983. The number of HIV-I infected children has increased in recent years in parallel with the increase in HIV-I infected women of childbearing age (Soeiro *et al.*, 1992). Although children have been infected by the inadvertent use of HIV-I contaminated blood or blood products, the overwhelming majority of childhood HIV-I infections, in the USA is believed to be the result of pre- or perinatal transmission from infected women (Fallon *et al.*, 1989). Studies have shown that the frequency of materno-fetal transmission of HIV-I varies from 12,9% to 65% (Soeiro *et al.*, 1992). In general, a higher frequency of mother-to-child transmission was observed in Africa (Dunn *et al.*, 1992; Lallemand *et al.*, 1994). The reasons for this apparently higher transmission rate in Africa are not clear, but it appears that two factors may be implicated. The first is advanced immunodeficiency in the mother, which increases the risk for transmission. It is likely that the higher viral load associated with immune depletion and with early infection is a major determinant of the degree of infectiousness, whether transmission is sexual or vertical. A second factor contributing to materno-fetal transmission is breast feeding (see chapter 1.7.1.5.3). Although mother-to-child transmission of HIV-II undoubtedly can occur, it seems less common and a much lower rate of transmission is observed than for HIV-I (Paulsen *et al.*, 1989).

There are three basic routes by which HIV-I can pass from mother to child: (1) transplacental (2) during delivery, and (3) postpartum (mainly through breast milk) (Bardequez and Johnson, 1994; Borkowsky and Krasinski, 1992; Dunn *et al.*, 1992; Sprecher *et al.*, 1986; Maury *et al.*, 1989).

1.7.1.5.1 Transplacental route

The role of the placenta in maternal-fetal transmission is poorly understood. The placental cells which are most likely to be susceptible to infection by the virus are the trophoblasts and the macrophages (Hofbauer cells) (Lewis *et al.*, 1990). Potential routes whereby HIV gains access to the fetal circulation include direct infection of the syncytio-trophoblast layer, infection of the Hofbauer cells or invasion through the villous stroma into the fetal circulation. It is possible that free virus or HIV-laden maternal lymphocytes could be transferred to the fetus. *In vitro* studies suggest that the placenta could become infected with HIV by the interaction of virus-infected maternal lymphocytes with syncytio-trophoblasts in direct contact with maternal blood in the intervillous space, while trophoblast cells exposed to cell-free HIV for up to 24 hours showed no evidence of virus uptake or replication (Douglas *et al.*, 1991).

In an attempt to localize HIV-positive cells in the placenta, immunoperoxidase techniques were used (Martin *et al.*, 1991). HIV p24 antigen was detected in villous Hofbauer cells, decidual macrophages, intermediate trophoblast and villous endothelium in four of nine placentas from HIV-infected women. Virological and histological evidence of HIV replication was found in about 25% of placentas obtained at term from HIV-infected women, but the presence of infection in the placentas did not correlate with infant infection status. Thus the role of placental macrophages in mother-to-child transmission of HIV remains unclear.

Theoretically, the macrophages can either protect the fetus from HIV infection, or serve to breach the placental barrier, at times acting as an effective reservoir of HIV-infection. Isolated case reports of early HIV disease in neonates give supporting evidence for transplacental transmission. Beach and co-workers (1991) reported the diagnosis of *Pneumocystis carinii* pneumonia in a 19 day old infant, implicating

transplacental infection causing immune compromise in the newborn. Evidence also exists for the role of the placenta in preventing transmission of HIV. Borkowsky and co-worker (1992) found a significant increase in the maternal-fetal transmission rate (80% vs 20%) of HIV in women who have experienced untreated syphilis during their pregnancies. Since *Treponema pallidum* is known to cause a placentitis, it was suggested that the placenta is fairly effective in preventing HIV transmission in the absence of a secondary infection. Cytomegalovirus (CMV) infection was monitored as a control pathogen, and was not recovered more often from women who transmitted HIV to their offspring than from those who did not. This suggests that the placenta probably constitutes an effective barrier to viral transmission unless disrupted by processes such as syphilis. Prevention of such breaks in the trophoblast barrier and efforts to stimulate maternal and newborn HIV-specific immunity may further decrease the perinatal transmission rate.

1.7.1.5.2 Intrapartum (transmission during delivery)

The large quantities of infectious blood and amniotic fluid present during delivery raises the possibility that, like hepatitis B, infection with HIV could occur around the time of delivery. HIV has been isolated from vaginal and cervical secretions from HIV-positive women (Vogt *et al.*, 1986; Henin *et al.*, 1993; Wofsy *et al.*, 1986).

In 1991, Goedert and co-workers investigated HIV infection rates in twins born to HIV-positive mothers. They showed a higher risk of HIV infection in first-born twins, with 25% of firstborns being infected compared to 8% of second-born twins. The data suggests that it is more likely for HIV infection to occur during the passage of the first twin through the birth canal. The European Collaborative Study Group (1994) assessed 1254 HIV-infected mothers and their children and the effects of different modes of delivery on transmission risk. Caesarean section was estimated to

halve the rate of transmission since those women who had caesarean sections were more advanced in their disease progression, the protective effect of caesarean section may be underestimated. These findings are important as they provide insight into the timing of infection from mother-to-child, as well as possible ways of preventing infection in the newborn.

1.7.1.5.3 Postpartum (mainly breast feeding)

The discovery of HIV-I in colostrum and breast milk (Thiry *et al.*, 1985; Dunn *et al.*, 1992; Hira *et al.*, 1990) raised the concern that postnatal transmission could occur during breast feeding. However, it was not initially considered to be a significant route of transmission because of the inhospitable environment of the infant digestive tract and the presence of anti-HIV antibodies in breast milk. Nevertheless, transmission via breast milk was increasingly documented all over the world (Hira *et al.*, 1990; Van de Perre, 1991; Ziegler *et al.*, 1985). The European Collaborative Study Group (1992) analysed the rate of vertical transmission, based on 721 children born to 701 mothers and found that the ratio of transmission was two-fold higher in breast-fed versus never-breast-fed children, despite the short (4 weeks) median duration of breast feeding. The balance of evidence was therefore in favour of HIV transmission through breast milk.

If infection of newborn babies occurs through ingestion of HIV-I, then there is an urgency to learn more about mucosal transmission. The question arises as to whether infection is initiated by cell-free virus or by HIV-I-infected cells in breast milk? Cell-free virus could penetrate the mucosal lining of the gastrointestinal tract of infants, either by infecting CD4⁺ monocytes, lymphocytes or Langerhans cells in the gut or by direct entry into the bloodstream via mucosal breaches. If HIV-I infection occurs only through cell-associated virus, then colostrum milk, which is richer in macrophages than later milk, would probably be more infectious. Transmission might be reduced by limiting breast feeding until after colostrum and early milk have been expressed and discarded (Van de

Perre *et al.*, 1993). The inhibitory qualities of human breast milk have recently gained considerable attention as a potential mechanism for the prevention of HIV infection. It was suggested that anti-HIV-I IgM in breast milk could be protective against postnatal transmission of the virus (Van de Perre *et al.*, 1993). In the developed world where resources are available, it might be possible to identify those HIV-I-infected women who are more likely to infect their infants through breast feeding, namely those with clinical AIDS, low CD4 counts at delivery or the presence of PCR-positive cells in early milk, together with the absence of HIV-I-specific IgM. Individual advice regarding the advisability of breast feeding could then be offered.

Thus, factors such as the inhibitory properties of breast milk, the development of an effective local immune response, the stage of clinical disease of the index case and/or the virus load (either as cell-free or cell-associated virus) appear to be important determinants of HIV transmission.

1.8 Disease In Infants

Paediatric AIDS is usually acquired perinatally from an HIV-infected mother. Most infected infants have no manifestations of the disease at birth, but 50% of HIV-infected children are symptomatic in the first 12 months of life and 78% have clinical disease by 2 years of age (Auger *et al.*, 1988, Blanche *et al.*, 1994). Most children with symptomatic HIV infection have several of the following symptoms, which are presented in descending order of frequency: Lymphadenopathy, hepatosplenomegaly, splenomegaly, poor growth, recurrent diarrhoea, more than 10% weight loss and, more rarely, parotitis. The most

debilitating of these is significant weight loss, or the "wasting syndrome", which may be life-threatening.

The majority of neurological manifestations of paediatric AIDS are believed to be the result of direct infection of the brain by HIV (Shaw *et al.*, 1985). A characteristic encephalopathy occurs that consists of developmental delay or deterioration of motor milestones, intellectual ability, or behaviour. The neurological disease may be static or slowly or rapidly progressive. The majority of symptomatic HIV-infected children have some degree of neurological involvement of which encephalopathy may be the dominant manifestation.

Lymphocytic interstitial pneumonitis (LIP) is a chronic interstitial pneumonia that results from diffuse infiltration of the alveolar septa and peribronchiolar areas by lymphocytes and plasma cells. The exact role of Epstein-Barr virus (EBV) in LIP is not clear, but it is likely that an interaction between HIV and EBV-infected B cells in the lungs may account for the pathogenesis of this condition. LIP occurs in approximately 40% of children with AIDS, but is quite rare in adults. The mean survival time of children diagnosed with LIP is 91 months (Turner *et al.*, 1993).

As in adults, *Pneumocystis carinii* pneumonia (PCP) is the most common opportunistic infection in children with AIDS, occurring most frequently in the first 6 months of life (Simmonds *et al.*, 1993). The high mortality associated with PCP is not diminished by intensive care or high-dose trimethoprim-sulphamethoxazole (TMP-SMX) and steroid therapy. Other important opportunistic infections include disseminated *Mycobacterium avium* complex infection, *Candida* esophagitis or pneumonia, disseminated cytomegalovirus or herpes simplex virus infections and intestinal cryptosporidiosis.

One of the more common problems associated with HIV infection in children is recurrent bacterial infections, such as sepsis, meningitis, pneumonia and soft tissue abscesses. Encapsulated organisms, such as *Haemophilus influenzae* and *Streptococcus pneumoniae* are most commonly observed. Recurrent bacterial infections occur in approximately 30% of children with AIDS, whereas similar repeated infections are considerably rarer in adults. Anti-bacterial immunizations are recommended for all HIV-infected children and should be given early in the course of the disease, since their efficacy is reduced in children with severe immune suppression (National Institute of Child Health and Human Development Intravenous Immunoglobulin Study Group, 1991).

The management of HIV-infected children will continue to evolve particularly with the increased use of PCP prophylaxis and the availability of anti-retroviral therapy. The challenge for the future is to prevent perinatal transmission, develop new and better therapies for opportunistic infections and HIV-associated complications and to improve outcome and prognosis.

Recent studies (Boyer *et al.*, 1994) have shown that zidovudine therapy can reduce HIV transmission from an HIV-infected pregnant woman to her baby by about two thirds. The analysis revealed a transmission rate of 2,5% when both mothers and babies received zidovudine in comparison with a transmission rate of 8,3% among those receiving a placebo. Because the long-term consequences of zidovudine therapy are still unknown, the investigators plan to follow these infants for several years. They also plan to follow the women in the trial for 6 months after delivery. Even if these long-term studies indicate other mortality risks, the benefits of substantially reducing the risk of HIV transmission from infected women to their infants, will likely outweigh them.

1.9 Epidemiology

1.9.1 Spread of HIV-I

The World Health Organization (WHO) has estimated that by mid-1993, over 13 million young people and adults would have become infected with HIV since the start of the pandemic, the majority through heterosexual intercourse, and that about 1 million children would have been infected perinatally. More than 8 million of the infections in adults have occurred in sub-Saharan Africa and over 1,5 million in North America and Western Europe, where the HIV pandemic is the oldest. Latin America and the Caribbean (1,5 million adult infections), North Africa and Middle East (75 000) and eastern Europe and central Asia (50 000) are showing a rise in HIV infections, with south and southeast Asia showing the steepest rises (over 1.5 million adult infections) (Merson, 1993). Up until the middle of 1993, over 2 million HIV infected adults have developed AIDS and most of them had died.

Currently there are four patterns of epidemiology which have evolved and been described for HIV infection and AIDS. These patterns are based partly on time, risk factors and modes of transmission (Piot *et al.*, 1992). The four patterns are as follows:

Pattern 1 - The extensive spread of HIV which began in the late 1970s or early 1980s. Most of these cases of AIDS occurred among homosexual and bisexual males and intravenous drug users. Heterosexual transmission is responsible for a small, but increasing, proportion of the total. This pattern is observed in the USA, most European countries and some Latin American countries.

Pattern 2 - Occurs mainly among heterosexuals. The male to female ratio is approximately one, and perinatal transmission is common. Most sub-Saharan and Latin American countries conform to this pattern.

Pattern 3 - Asian, north African and Oceanic countries fall into this category. Most of the cases have been introduced into the countries from elsewhere and there is no predominant mode of transmission.

Pattern 4 - This pattern occurs in countries where there was first a predominantly homosexual transmission, later becoming heterosexual, with very few intravenous drug abusers. Some countries in the Caribbean basin fall into this group.

A few countries in the developed world are showing trends that may change their pattern from 1 to 2 because of increased heterosexual transmission. In South Africa, patterns 1 and 2 are observed.

1.9.2 AIDS in Africa

1.9.2.1 Central African Region

Cameroon, Congo, Central African Republic, Equatorial Guinea, Gabon, Zaire, Rwanda and Burundi comprise the Central African Region. These countries can be divided into 3 groups, A, B and C, based on HIV-I and HIV-II seroprevalence rates in each country. Group A countries (Zaire, Congo, Central African Republic, Rwanda and Burundi) are the most severely affected. The two countries (Group B) which appear to be moderately affected in the high risk urban populations are Gabon and some parts of the Republic of Cameroon. Equatorial Guinea (Group C), a country with very little data, appears to have minimal infection (Williams, 1992).

1.9.2.2 East African Region

The nine countries which belong to the East Africa Region are: Ethiopia, Djibouti, Somalia, Sudan, Kenya, Uganda, Tanzania, the Comores and the Seychelles. Here it is noteworthy that the seroprevalence rate is higher in

the southern portion of Sudan where there is ongoing civil strife. It is also in closer geographic proximity to Ethiopia, Uganda and Kenya, where up to 86% of urban prostitutes and barmaids have been shown to be HIV-I seropositive (Piot *et al.*, 1987).

1.9.2.3 Southern African Region

Mozambique is the largest country in land mass in this region, but has one of the lowest published seroprevalence rates for HIV-I in southern Africa. This is in contrast to the relatively high prevalence rates for hepatitis B infection and liver cancer in that country. HIV-II, however, is not uncommon in Mozambique. Malawi, Zimbabwe and Angola appears to have moderate rates of infection while Zambia is the only severely affected country in southern Africa. In South Africa, the Department of Health reported more than 8500 confirmed AIDS cases by October 1995 (Table 2).

Table 2 Reported AIDS cases in South Africa as on 17 October 1995 (Department of Health, RSA, 1995)

AIDS in South Africa											
Reported AIDS cases as on 17 October 1995											
Number of AIDS cases reported by region											
Province	82-86	87	88	89	90	91	92	93	94	95	Total
Eastern Cape (EC)	-	-	1	4	10	32	69	146	255	19	536
Western Cape (WC)	12	2	20	32	39	44	63	86	85	40	423
Northern Cape (NC)	-	-	1	1	3	10	10	64	73	1	163
KwaZulu-Natal (KN)	4	5	19	37	140	171	352	865	1912	625	4130
Free State (FS)	1	2	4	9	8	38	45	60	421	322	910
Gauteng (GT)	29	31	44	92	118	153	253	174	82	10	986
Mpumalanga (MP)	-	-	1	2	9	64	40	120	188	53	477
North West (NW)	-	-	1	-	3	5	16	78	255	113	471
Northern Province (NP)	-	-	1	1	5	2	7	128	229	102	475
Total SA	46	40	92	178	335	519	855	1721	3500	1285	8571
Other countries	12	9	6	3	8	20	16	96	55	4	229
Unknown	-	-	-	-	-	-	-	2	-	-	2
Total	58	49	98	181	343	539	871	1819	3555	1289	8802

1.9.2.4 North African Region

The seroprevalence rates for HIV-I in this region (Egypt, Libyan Arab Jamahiriya, Tunisia, Algeria, Morocco and Saharawi Arab Democratic Republic) are generally low (Giasudden *et al.*, 1988), but relatively high among the high-risk groups of homosexuals, drug abusers and prostitutes who do not practice safe sex. All these countries belong to "Pattern 3" of the WHO epidemiological classification.

1.9.2.5 West African Region

Ivory Coast, which has its capital Abidjan, is the only West African country that has recently and suddenly become severely affected by both HIV-I and HIV-II infections (De Cock *et al.*, 1989). There is a predominance of infected males, over females, for both types of HIV. Cape Verde consists of a group of islands and belongs to the West African Region. HIV-II is relatively common in this area because of its proximity to Senegal and Guinea Bissau, where HIV-II was first identified and where its prevalence is relatively high (Clavel *et al.*, 1986). Some of the islands, e.g. Sal Island, seem to have escaped the HIV epidemic because there have been no detectable or reported HIV-I or HIV-II infections so far.

1.9.3 Worldwide epidemiology

Sub-Saharan Africa is home to less than 10% of the world's population, yet it has almost 70% of the world's total of HIV infected people and over 90% of the world's HIV infected women and children. HIV infection is now spreading rapidly in some countries in Asia, especially Thailand and India (De Cock, 1993).

The highest rates of HIV infection among asymptomatic persons in developing countries are found in female prostitutes, in whom rates in excess of 80% have been described in some African cities. Blood donors and pregnant women, who have no special risk for HIV infection, have an HIV infection rate of about 10% in 10 major cities of sub-Saharan Africa. Heterosexual transmission is rapidly becoming the dominant mode of spread and the driving force behind the epidemic in most countries. Since most HIV infections in children occur as a result of perinatal transmission, the childhood infection rate is directly related to that in adult women.

1.10 Anti-viral drugs and vaccination

1.10.1 Anti-viral drugs

At present, no cure exists for any retroviral infection, including HIV. However, in 1985 it was shown that 3'-azido-3'-deoxythymidine (AZT) inhibits the replication of HIV-1 *in vitro* (Mitsuya, 1985). Zidovudine is a synthetic pyrimidine analog of AZT with its target being the reverse transcriptase enzyme which is essential for viral DNA replication and is unique to retroviruses (Furman *et al.*, 1986). Phosphorylation of zidovudine to its active form, AZT-5'-triphosphate (AZT-TP) is accomplished by cellular enzymes. The AZT-TP form binds preferentially to HIV-1 reverse transcriptase, and the incorporation of azidothymidilate into the growing DNA strand leads to chain termination. Clinical trials that were undertaken in 12 different centers in the USA showed a decreased rate of mortality in AIDS patients who were treated with AZT. Individuals who received AZT generally showed an increase in peripheral blood CD4 cells and also showed a general weight gain compared to placebo recipients who lost weight (Fischl *et al.*, 1987). The treatment with AZT

did, however, produce some side effects. Macrocytic anaemia occurred frequently in the AZT group and made transfusions necessary. Another side effect was neutropenia, with headache, nausea, myalgia and insomnia also being reported more frequently in recipients of AZT. These side effects were reversible and generally tolerable for patients with AIDS or severe ARC. The currently recommended dosage of AZT for adults is 200mg every 4hr (Hirsch and Curran, 1992). Other nucleic acid analogs that also target the enzyme, reverse transcriptase, include dideoxyinosine (ddI) and dideoxycytidine (ddC). These two drugs which have now been approved for therapy by the Food and Drug Administration (FDA) appear to be effective and less toxic to bone marrow than AZT (Butler *et al.*, 1991 and Yarchoan *et al.*, 1988). Some studies have also suggested that it could be helpful to use ddI or ddC in combination with AZT. A recent report (Khan *et al.*, 1992) suggested that changing treatment from AZT to ddI in symptomatic individuals with low CD4⁺ cell counts could slow the progression of disease.

There has, however, been one notable problem in AZT therapy, namely the emergence of resistant virus strains. Resistance to ddI and ddC has also been reported (St. Clair *et al.*, 1991; Gao *et al.*, 1992). Thus far, most data suggest that the use of anti-reverse transcriptase drugs alone will help to reduce and delay the symptoms of HIV infection. Nevertheless, because of the rapid development of drug resistance, other therapies must be found that can be used either alone or in combination. This latter approach offers the best chance of success for permanent control of HIV infection.

Other approaches to antiviral therapy include blocking of the cellular virus receptor with recombinant CD4, or blocking virus attachment with sulfated polysaccharides, the use of interferons and strategies that destroy the virus-infected cells (Baba *et al.*, 1988 and Poli *et al.*, 1989).

Attempts at limiting virus replication have involved molecular procedures directed at inhibition of the viral protease or integrase. Protease-inactivating analogs have been tried and certain selected compounds await clinical trials, but resistance to these drugs could also emerge (Dreyer *et al.*, 1989).

1.10.2 Vaccines

Several features of HIV infection and transmission must be considered in vaccine work. They include the appreciation of viral heterogeneity, the potential for autoimmune responses, local mucosal immunity and virus transmission by infected cells (Levy, 1988 and Forrest, 1991). The ideal properties of a vaccine are listed in table 3.

Table 3 Desirable properties of a HIV vaccine

Elicitation of neutralizing antibodies to all HIV strains and subtypes
Induction of humoral and cellular immune responses against virus-infected cells
Induction of immune responses that recognize latently infected cells
No induction of antibodies that enhance HIV infection
No induction of autoimmune responses
Induction of local immunity at all sites of HIV entry in the host
Safe with no toxic effects
Long-lasting effect

A number of different strategies are being pursued for the development of efficacious AIDS vaccines. These different strategies include targeting of the immune response to specific HIV-I B- and T-cell epitopes, and the development of candidate vaccines that mimic attenuated or inactivated HIV-I or are designed to induce an anti-HIV mucosal immune response.

Various obstacles have hampered the development of an effective vaccine against HIV-I. A major problem has been the lack of an appropriate animal model. Chimpanzees are expensive and do not replicate HIV to high titer or contract disease. Studies with simian immunodeficiency virus (SIV) have been helpful but may not always give results that are directly comparable with human lentiviruses. Recent reports showed that one macaque species, *Macaca nemestrina*, could also be infected with HIV-I, but this requires further confirmation (Agy *et al.*, 1992). The high degree of genomic diversity and the rapid rate of viral mutation exhibited by HIV-I poses the second hurdle to vaccine development. A segment that codes for the third hypervariable region (V3) of the envelope glycoprotein gp120, that contains the principle neutralization domain (PND), exhibits the highest degree of heterogeneity (Putney *et al.*, 1986; Javaherian *et al.*, 1990 and Fisher *et al.*, 1988). Because of significant geographic diversity, vaccine candidates must be designed to incorporate immunogenic epitopes conforming most closely to HIV-I isolates prevalent in areas where the vaccine is to be used. Strains of HIV-I, as well as HIV-II and SIV, are under study by the Theoretical Biology and Biophysics Group of the Los Alamos National Laboratory (Myers *et al.*, 1992). Nucleotide and amino acid sequence databases of the *env* and the more conserved *gag* gene have been established. A third obstacle stems from the life cycle and mode of transmission of the virus. As previously mentioned, HIV-I can infect several cell types and may establish a state of latent infection during which the integrated proviral DNA does not express proteins that would

otherwise be recognized by the host immune system. Also, transmission of HIV-I infection can be initiated by either a cell-free virus or virus-infected cells. Thus a vaccine capable of eliciting both neutralizing antibodies and cell-mediated immune response would likely be the best prototype vaccine (Mann *et al.*, 1990).

Finally, it has been impossible so far to establish a definitive biological correlate of immunity since the viral components/epitopes that induce protective immune responses have not yet been unambiguously delineated. The various approaches to HIV vaccines are listed in table 4.

Table 4 Different approaches to potential HIV vaccines

Whole inactivated virus
Attenuated (nonpathogenic) variants
Subunit vaccine using the envelope glycoprotein (gp120)
Subunit vaccine using the transmembrane glycoprotein (gp41)
Subunit vaccine using the gag protein
Viral proteins in other vectors (eg vaccinia virus, poliovirus or baculovirus)
Sequence-derived peptides of HIV

The initial vaccine studies with Lentiviruses that showed some virus protection, involved the use of killed SIV in macaques (Desrosiers *et al.*, 1989; Murphy *et al.*, 1989). Protection against low-dose challenge with the homologous SIV strain were demonstrated by this approach. However, studies have also shown that killed-virus vaccines can protect against infection by intravenously delivered challenge dose but not against virus inoculated onto the mucosal lining of the genital tract

(Sutjipto *et al.*, 1990). Immunization with a live attenuated SIV_{mac} strain have been explored and shown to prevent disease, but not to prevent infection, by a virulent challenge strain. As a model, the use of an attenuated agent comes closest to resembling natural infection and these studies should be continued to indicate whether conventional vaccine approaches with live attenuated virus would induce at least protective immunity against an agent such as HIV (Marthas *et al.*, 1990).

Limited success has been achieved with a vaccine comprising purified envelope glycoproteins expressed in the baculovirus system or in mammalian cells. In a trial with chimpanzees, immunization with the purified HIV gp120 prevented infection after a low-dose HIV-challenge (Berman *et al.*, 1990). More recently, chimpanzees that were multiply immunized with HIV-I antigens showed resistance to intravenous challenge with infected PBMCs. This study provided the first evidence that immunization can induce host responses against HIV-infected cells (Fultz *et al.*, 1992).

The use of viral proteins and not whole virus as immunogens eliminates the possibility that viral nucleic acids will become transcribed or undergo cellular integration even though the virus has been killed. A killed-virus preparation cannot guarantee stability of expression (antigenicity) of all the viral proteins or exclude the possibility of residual infectivity.

Potential AIDS vaccines are currently being evaluated in uninfected human volunteers for their safety and immunogenicity. A large number of these experimental vaccines are genetically engineered immunogens, mostly recombinant HIV-I envelope glycoproteins gp160 and gp120 (Picard *et al.*, 1990 ; Redfield *et al.*, 1991).

Chapter 2

Diagnostic assays for HIV-I

2.1 Serological diagnosis

2.1.1 Antibody assays

The diagnosis of HIV-I infection is generally based on the identification of specific anti-viral antibodies. These become detectable from as early as four to six weeks after exposure to the virus (see Fig. 5).

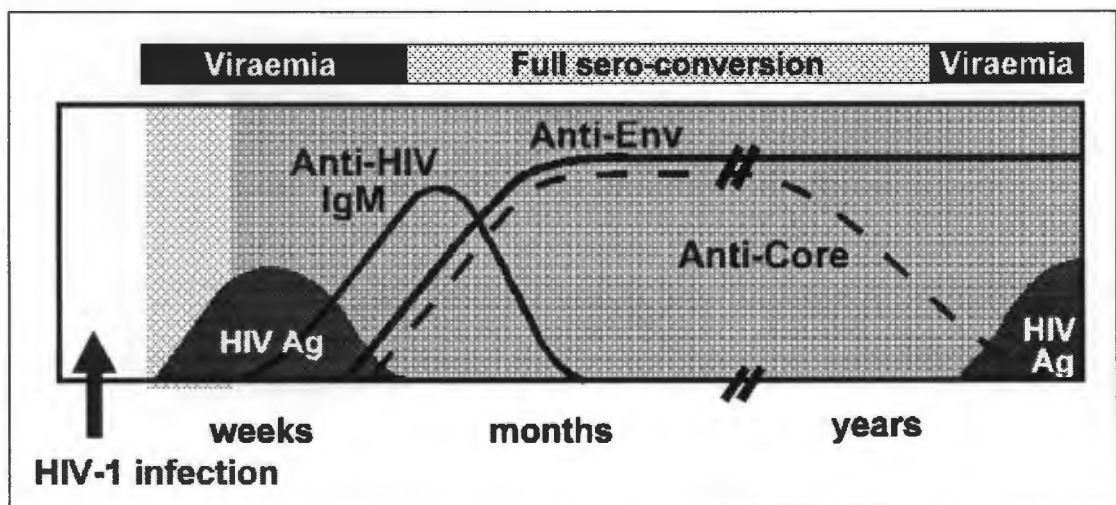


Fig. 5 Serological profile of HIV-1 infected patients (excluding perinatally infected infants). Viral antigen is elevated after initial infection, falls to low levels during the asymptomatic stages and becomes elevated again as symptoms develop and the disease progresses (Adapted from Rogers *et al.*, 1991).

Commercial assays were first licensed by the Food and Drug Administration (FDA) in 1985 (Hou *et al.*, 1994). The enzyme linked immunosorbent assay (ELISA) is the most commonly used serological test (Weiss *et al.*, 1985). In principle, HIV-1 antigens are bound to a solid

phase. Samples of patients' sera are incubated with the solid phase for 1-2 hours and if antibodies to HIV-I are present they will bind to the antigens. After washing to remove any unbound material, enzyme-linked anti-human IgG antibody conjugate is added to the solid-phase antigen-antibody complex and incubated for a period of time. After a second wash to remove excess unbound antibody, enzyme substrate is added, resulting in colour development. The intensity of the colour reaction correlates with the amount of anti-HIV-I antibody in the sample and can be quantitated by using a spectrophotometer.

Early first generation ELISAs used a preparation of HIV-I antigens harvested from infected T4 lymphocytes. This partially purified HIV-I antigen standard reacted with antibodies to proteins of the viral envelope, core and polymerase, that may be present in test sera. These first generation antigen standards, however, were often contaminated with non-viral antigens, such as those of the major histocompatibility locus originating from the lymphocytes. The resulting cross-reactivity produced a relatively high rate of false-positive results. Soon thereafter, second generation (Fig. 6) tests were developed in which the viral antigens were either (i) recombinant, i.e. produced by the insertion of a portion of a viral genome into a vector (bacterial or yeast) that is easily cultured to generate large quantities of specific viral antigens, or (ii) synthetic, i.e. chemically synthesized peptide chains, 10 to 40 amino acids long, that are homologous in sequence to a portion of a viral antigen.

Tests based on either recombinant or synthetic peptide antigens have sensitivities and specificities higher than first-generation tests. However, recombinant antigen tests may also yield some false-positive results due to contaminants from the vector, and the synthetic antigen tests may lack sensitivity. The generally excellent specificity and sensitivity of commercially available second generation test kits have been evaluated

and reviewed several times, sensitivity should approach 100% and specificity should be above 97%.

In the third generation tests that are now available, enzyme-labelled recombinant or synthetic peptide antigens have been employed in the detection step, creating “sandwich” ELISAs, with further gains in sensitivity and specificity (Fig. 6). All classes of immunoglobulin antibodies are detected, thereby allowing earlier recognition of infections, and the window period between infection and seropositivity is shortened by an average of 5 days, the median length of the window having been estimated previously to be 6 weeks (Horsburgh *et al.*, 1989).

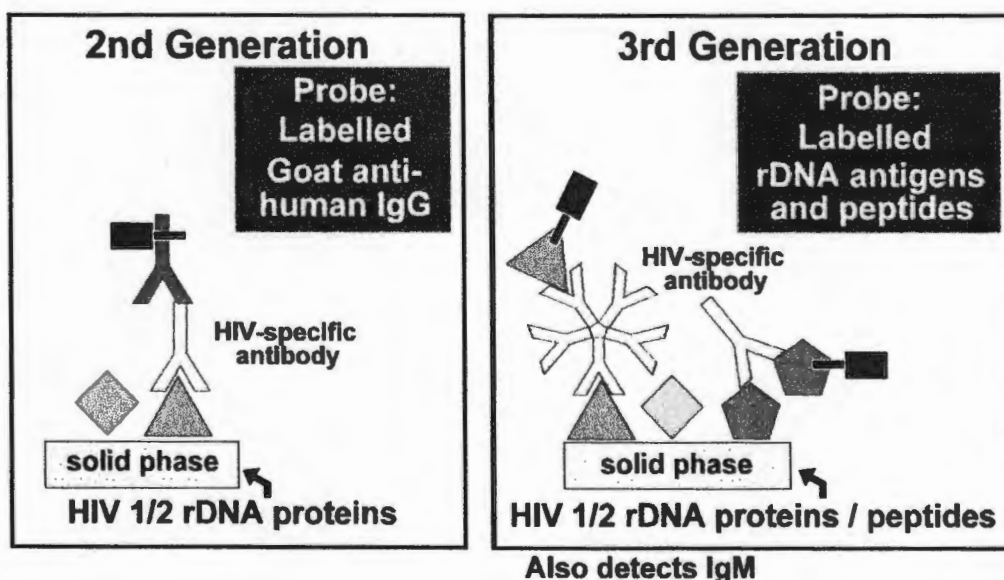


Fig. 6 Second and third generation antigen-sandwich ELISAs for detection of immunoglobulin antibodies to HIV-I, -II.

The conventional method of anti-HIV testing has been to apply a screening test in which all negative results are accepted as true, but repeatedly positive sera are subjected to an additional confirmatory assay, usually the western blot (WB) assay, or an equivalent test. The WB procedure enables the detection of antibodies to individual viral antigens, such as viral core proteins (p24, p55 and p17) and envelope glycoproteins (gp120, gp160, gp41). Viral proteins are first separated into well-defined

bands by electrophoresis through agarose gel. The separated bands are transferred or 'blotted' onto a nitrocellulose membrane that is cut into strips and sequentially exposed first to the patient's serum sample and then to an anti-human antibody conjugated to an enzyme. Serum antibodies to the antigen standards are detected as discrete coloured bands (Fig. 7). The diagnostic pattern of bands identified in the WB is more specific than the ELISA for viral antibodies.

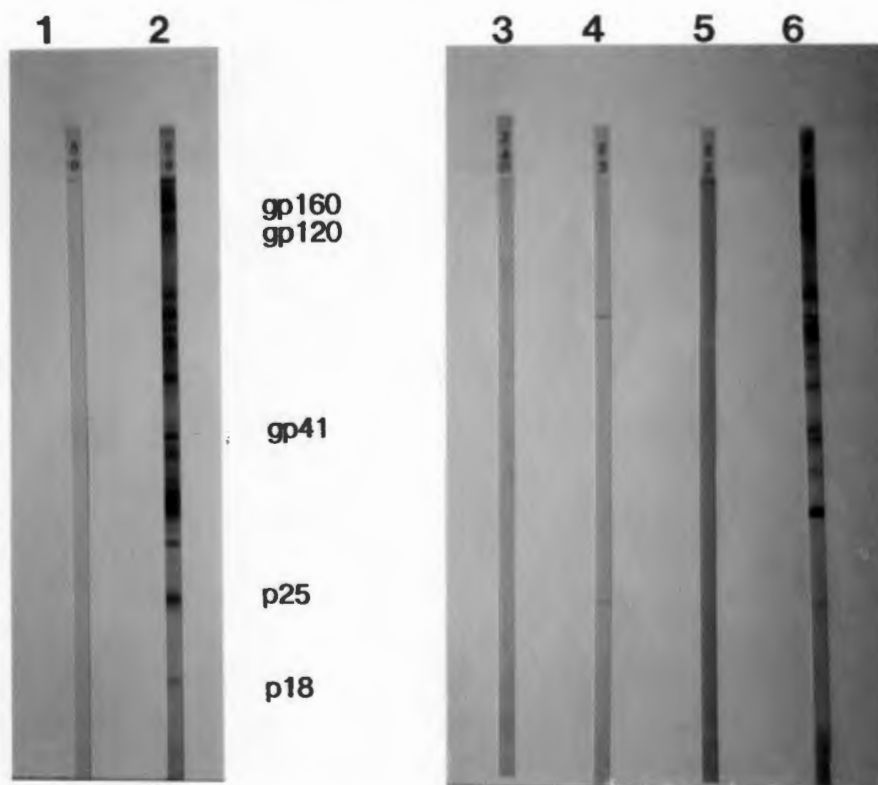


Fig. 7 Solid phase Western blot assay for the detection of anti-HIV-I antibodies. The negative test control is shown in lane 1, and the positive test control is shown in lane 2. Four patient sera were evaluated in lanes 3 to 6, with lane 3 showing a negative result; lane 4 an indeterminate result; lane 5 another negative result and lane 6 a positive result (Department of Virology, UCT, Medical School).

The WB assay has the disadvantage of being expensive, technically complicated and difficult to interpret. The Center for Disease Control (CDC) standard requires that at least two of the three main structural antigen bands (p24, gp41 and gp120/160) must be present before a

sample may be designated as positive for antibody to HIV-I. Blood specimens that do not meet the two-band requirement for WB but still exhibit some of the bands characteristic of HIV-I proteins are said to have an 'indeterminate' pattern (Jackson *et al.*, 1990; Genesca *et al.*, 1989). Such patterns are seen in three categories of patients: - those in the early stages of HIV-I infection, those infected with a closely related retrovirus (e.g. HIV-II) and truly false-positive results, i.e. healthy people who are not infected with HIV-I or HIV-II. For those in the early stages of HIV-I infection and also for those with HIV-II infection, one of the first HIV antibodies detectable by WB is the antibody to the viral core protein p24. Most people infected with HIV-I will eventually develop the other HIV-I antibodies within 6 weeks (Horsburgh *et al.*, 1989).

Testing for HIV could be simplified by the application of first one ELISA for screening, followed by a second different ELISA for confirmation (the latter replacing WB). The two tests should be based on different principles (e.g. indirect and competitive ELISAs) and have different sources of antigen (e.g. viral lysate and synthetic peptides). The first test should have high sensitivity, be technically simple and inexpensive, while the second test should have high specificity. Only the few sera giving discrepant or indeterminate results need to be referred for WB analysis, resulting in considerable savings in costs.

2.1.1.1 Sensitivity and specificity of antibody tests

A test with a high sensitivity will have few false-negative results. Therefore, only tests of the highest possible sensitivity should be used when there is a need to minimize the rate of false-negative results (e.g. in testing of blood/organ donations). A test of high specificity will have few false-positive results and should be used when there is a need to

minimize the rate of false-positive results (e.g. in diagnosis of HIV infection) (Meissner and Coffin, 1992).

2.1.2 Elispot

The technique of *in vitro* antibody production (IVAP) is based on the principle that B-cells in HIV-I infected patients are usually activated *in vivo* and hence spontaneously secrete anti-HIV-I antibody (Pahwa *et al.*, 1989). Peripheral blood lymphocytes from infected individuals may be isolated and cultured *in vitro* and activated by mitogen such as pokeweed. *In vitro* antibody production involves coculture of mitogen-stimulated patient peripheral blood mononuclear cells (PBMCs) with normal healthy donor lymphocytes. The presence of HIV-I is confirmed by periodically testing the culture supernatant for the presence of anti- HIV-I antibody (De Rossi *et al.*, 1988; Pahwa *et al.*, 1989). The ELISPOT test involves incubating antibody-producing patient PBMCs in polystyrene wells that have been coated with HIV-I antigen. In theory, exposure of the patient PBMCs to antigen results in production of detectable anti- HIV-I antibody. After the sample has been incubated and excess unbound antibody has been washed out, enzyme-linked anti-human IgG is added, and the presence of anti- HIV-I antibody is confirmed by detection of 'spots' at the bottom of the well, where the enzyme substrate had reacted.

2.1.3 Serological assays for the early diagnosis of HIV infection in infants <18 months of age

Serodiagnosis of HIV infection is more difficult in infants and young children than in adults. As a result of placental transfer of maternal IgG

antibody to HIV-I, virtually all neonates born to seropositive women are HIV-I antibody-positive at birth, and this maternally derived antibody may persist for up to 18 months of age (European Collaborative Study, 1988; Rogers *et al.*, 1991). Testing of serial samples of blood from uninfected infants by the WB technique may reveal a sequential loss of viral bands, representing the decline of maternal antibody, but this requires at least several months of observation. Because maternal IgM does not cross the placenta, the presence of specific IgM antibodies could be used to indicate the presence of HIV infection in infants. However, there is no consistent IgM antibody response by the infected infant to enable diagnosis of prenatal infection. Furthermore, available methods to detect HIV-I specific IgM antibody have a poor sensitivity (Pyun *et al.*, 1987). Assays for the detection of specific IgA antibody seem to offer a promising method of diagnosis, but these still require additional studies (Weiblen *et al.*, 1990). A small proportion of HIV-infected children are hypogammaglobulinemic, leading to false negative antibody tests once the maternally derived antibody has been catabolized (Borkowsky *et al.*, 1987).

In summary, present data on serum immunoglobulins in infants of seropositive mothers have shown that: (i) hypogammaglobulinemia (total IgG, IgA and IgM) is a common but relatively non-specific early finding in HIV-I infected infants, (ii) infected infants with advanced symptomatic disease may exhibit hypogammaglobulinemia presumably due to severe impairment of B-cell function, (iii) serial follow-up of anti-HIV IgG is an inexpensive and acceptable method for diagnosing HIV-I infection in infants, but the long duration of maternal antibody prevents early diagnosis, (iv) testing for anti-IgM or -IgA is relative specific but may not be adequately sensitive in young infants, (v) no information exists on anti-HIV-I IgA and -IgM testing in infants less than 3 months of age, and (vi) no

data regarding the sensitivity and specificity of the ELISPOT assay in very young paediatric patients exist, especially for infants under 3 months.

2.2 Antigen assays

An ELISA assay for the detection of HIV antigen was first described by McDougal *et al.* (1985). High-titer anti-HIV IgG, purified from a seropositive individual, was used in a sandwich ELISA test format. The IgG (used as capture antibody) was bound to the plastic surface of a microtitre well. HIV-I culture supernatant fluid was added, followed, after incubation and washing, by incubation with an enzyme-labelled human anti-HIV antibody. If antigen was present, it allowed the formation of a "sandwich" of capture antibody, HIV antigen and enzyme-labelled probe antibody. The addition of enzyme substrate resulted in colour development, which revealed a positive reaction. These initial tests were used to detect HIV antigens in virus culture fluids. The sensitivities were not adequate for the detection of the very low titers of HIV antigen present in body fluids of most HIV-infected individuals. The subsequent development of a triple-antibody sandwich procedure, that used antibodies primarily specific for the p24 antigen, provided the needed amplification to detect HIV antigens in clinical specimens (Goudsmit *et al.*, 1986). Several HIV antigen ELISAs are now commercially available. Serum, plasma and cell culture fluids may all be tested. The sensitivity of the HIV antigen assays in detecting HIV infection directly in patient specimens varies from 4% in asymptomatic seropositive subjects to 70% in patients with AIDS (Kenny *et al.*, 1987). The apparent lack of sensitivity in detecting HIV infection, limits its use as a screening assay for HIV infection in low-risk groups.

2.2.1 Antigen assay for the diagnosis of infected infants

Studies that compare the sensitivity of antigen detection with that of virus culture and the polymerase chain reaction assay (PCR) have shown that the antigen test is the least sensitive for the diagnosis of HIV-I in infants (De Rossi *et al.*, 1988; Krivine *et al.*, 1990). Krivine and co-workers reported a 96% concordance rate between viral culture and PCR results in PBMCs from 45 children born to seropositive mothers, however, p24 antigen could be detected in only 50% of virus culture-positive infants, and in only 44% of PCR-positive infants. It was shown that the antigen assay could give false positive results with blood samples from infants in the first 2 months of life (De Rossi *et al.*, 1988).

Recent studies have shown that detection of HIV-I p24 antigen may be enhanced by pre-treatment of samples with acid (Nishanian *et al.*, 1990). This is achieved by incubating serum samples with hydrochloric acid (HCl) at a pH of 2.5 to 3.0. Acid pretreatment dissociates antibody-antigen complexes and thereby releases the antigen enabling it to react in the ELISA which follows. Miles and co-workers used glycine hydrochloride to dissociate immune complexes, and they report that this is a simple serological procedure that may be of value during the diagnosis of HIV infection in neonates born to HIV infected women (Miles *et al.*, 1993).

2.3 Viral Culture Methods

2.3.1 Virus culture

Barre-Sinoussi and co-workers successfully isolated HIV-I in 1983. In 1984 Gallo and co-workers successfully cultured HIV-I by using HTLV9 cells from a neoplastic T-cell line. Since that time, virus isolation from the

cellular component of blood, plasma, body fluids, amniotic fluid and tissue has been reported (Coombs *et al.*, 1989; Ho *et al.*, 1989; Mundy *et al.*, 1987; Lewis *et al.*, 1990).

Recent advances have refined virus isolation techniques, greatly improving their sensitivity and specificity. Patient PBMCs are harvested from heparinized blood by Ficoll-Hypaque gradient separation. The PBMCs are co-cultivated with healthy donor mitogen-stimulated PBMCs in culture medium (RPMI 1640, supplemented with 20% fetal calf serum and interleukin-2) at 37°C in a 95% air - 5% CO₂ environment. The co-cultures are maintained by the periodic addition of interleukin-2 and fresh mitogen-stimulated healthy donor PBMCs. Culture fluids are collected periodically (about every 7 days) and tested for either HIV p24 antigen or reverse transcriptase. Cultured cells may also be examined by light microscopy for syncytium formation, although this criterion is not as specific as testing for viral products. Cultures that have no demonstrable evidence of HIV-I infection by 42 days are usually terminated. In early reports, the recovery rate by culture of virus seropositive patients ranged from 70 to 90% (Levy and Shimabukuro, 1985). The ability to culture virus from an individual may reflect that person's viral load. Newer improved culture methods can isolate HIV-I from about 97% of antibody-positive people (Jackson *et al.*, 1988).

Truly quantitative methods for viral culture have been reported by Ho and co-workers (1989). In this study, the patients' PBMCs were diluted in a 10-fold series and then co-cultivated with a standard number of uninfected PBMCs. The highest dilution of PBMC that yielded a positive culture was considered as the end point of the assay. The quantity of infectious HIV-I was reported in tissue culture infective doses per number of PBMCs. This study showed asymptomatic but infected patients to have a 100-fold lower tissue culture infective dose than patients with AIDS or AIDS-related

complex (ARC). These findings imply that asymptomatic patients have a lower viral burden than symptomatic ones.

Due to the risk to laboratory workers and the expense of HIV-I isolation, it is unlikely that culture methods will play a significant role in HIV-I testing, except in research settings.

2.3.2 HIV-I Culture for diagnosis of infected infants

Little data is available on recovery rates of virus from infants less than 3 months of age. One study estimated the percentage of culture positive samples from infants tested very early in life (less than 3 months of age) to be approximately 50% (Rogers *et al.*, 1991). This poor sensitivity of viral culture for the detection of HIV-I improves when older infants are tested. The poor sensitivity in the neonatal period (less than 1 month) may be due to several factors: (i) initial exposure to HIV-I may have occurred during delivery and the HIV-infection may not yet have progressed to the level of detection and (ii) even if infection occurs *in utero*, the quantity of circulating cell-free or cell-associated virus in the infant may be below the level of detection by culture. The application of quantitative culture methods may provide some benefit for newborns and infants with HIV-I infection: - for identifying infected infants at risk of rapid progression to symptomatic disease; for monitoring the natural progression of HIV infection in infants; and for assessing improvement after anti-retroviral therapy.

The rate of successful recovery of virus by culture probably correlates with the amount of virus present in the infected patient (or infant).

2.4 Polymerase Chain Reaction

One of the most promising techniques for the diagnosis of HIV infection is the polymerase chain reaction (PCR) which can be used to detect either proviral DNA sequences or genomic RNA sequences of HIV. The technique was initially developed in the 1980s and was first applied in the prenatal diagnosis of sickle cell anemia (Mullis and Faloona, 1987; Mullis, 1990; Embury *et al.*, 1987). Adaptation and application of PCR techniques for the detection of HIV can result in numerous advantages: (i) the ability to amplify gene sequences with very low copy number would enhance the sensitivity of detection, (ii) detection of HIV proviral DNA could represent an earlier marker of infection than detection of antibody, and (iii) although an HIV infection in a patient whose B-cell function is impaired may not be identified serologically, it could be detected by this method (Jackson *et al.*, 1990).

2.4.1 PCR - "The three step cycle"

The method is based on a three-step cycling process (Fig. 8) (Schochetman *et al.*, 1988; Mullis and Faloona, 1987; Embury *et al.*, 1987). The typical set of three steps (denaturation, annealing and extension) is referred to as a cycle. Multiple cycles result in an exponential increase in the number of copies of the target sequence. During the late PCR cycles, a reduction in the exponential rate of product accumulation occurs and this phenomenon is referred to as the "plateau effect". Optimizing the number of PCR cycles is the best way to avoid amplification of background products which accumulate after maximum amplification.

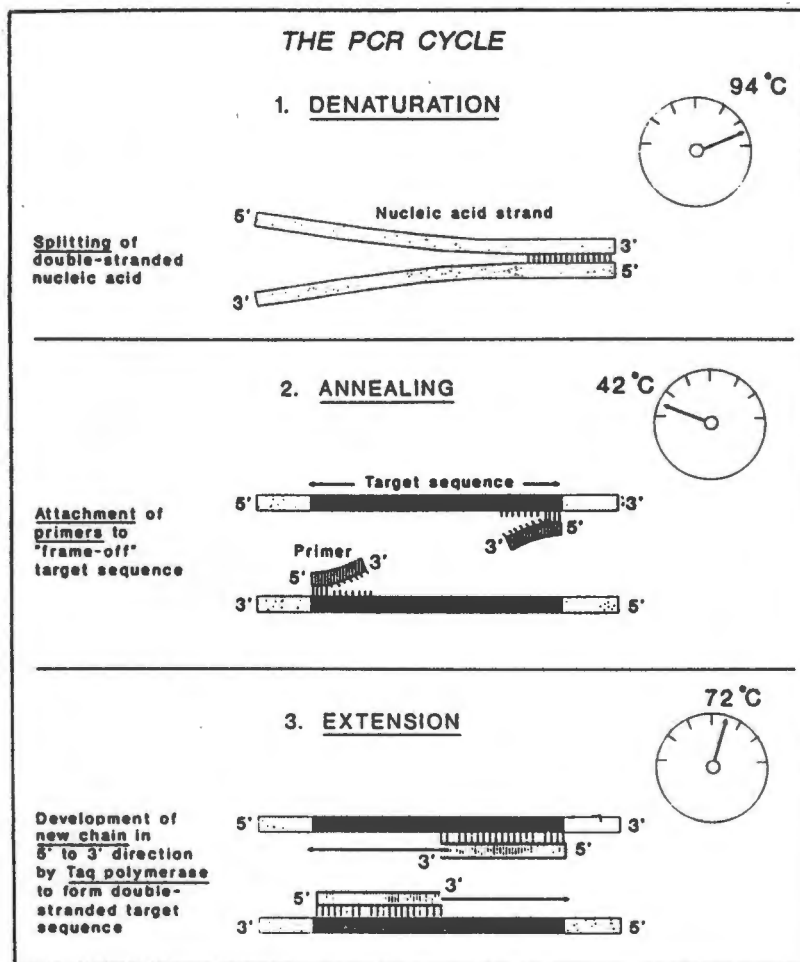


Fig. 8 The Polymerase Chain Reaction Cycle (Medicine Group, SA, 1992)

2.4.1.1 Denaturation

High temperature denaturation of double-stranded template DNA is the first step in the cycling process. Of the several physical and chemical means of dissociating complementary strands of DNA, heating (95°-100°C) is simple and efficient. The two strands will remain free in solution until the temperature is lowered sufficiently to allow annealing to take place. Ribonucleic acid can also be used as a template for amplification following the production of complementary DNA (cDNA) using reverse transcriptase (Ottman *et al.*, 1991).

2.4.1.2 Annealing

In this step, the temperature is lowered to 40°C - 60°C to permit annealing between the oligonucleotide primers and template DNA. Specific primers are synthesized according to the sequence of the DNA template at the boundaries of the region to be amplified. Since the primers anneal to opposite strands, they can be viewed as having their 3' ends facing in opposite directions. Typically, the primers have different sequences and are not complementary to one another. The primers are present in large excess over the DNA template, and when the temperature is lowered the formation of the primer-template complex will be favoured over the re-association of the two DNA strands.

2.4.1.3 Primer extension

The third step in the procedure is a DNA polymerase-mediated (5' to 3') extension of the primer-template complex. The temperature is raised to allow optimal activity of the DNA polymerase which is now able to extend each of the primers thus replicating the region between them. Initially, the PCR technique used the Klenow fragment of DNA Polymerase I of *Escherichia coli* with an extension step conducted at 37°C (Embury *et al.*, 1987). The thermal lability of the Klenow fragment required the addition of fresh enzyme after each denaturation step, which rendered the whole process very tedious and resulted in the rapid accumulation of denatured enzyme in the sample. Using a thermostable DNA polymerase purified from *Thermus aquaticus* (*Taq*) greatly simplified the whole procedure because the addition of fresh enzyme after each denaturation step was no longer required (Saiki *et al.*, 1988). The *Taq* polymerase allowed automation of the PCR process because all the initial reaction components now did not need to be replenished. Furthermore, the *Taq* polymerase has enabled DNA amplification to be performed at higher temperatures thus improving the specificity of the reaction (Saiki *et al.*, 1988).

2.4.2 Detection of PCR product

Detection of the amplified PCR product is usually achieved by hybridization with an oligonucleotide probe complementary to the original segment of HIV genome that has been amplified. Labelling of the probe can be achieved with different substances, e.g. enzymes (peroxidase or alkaline phosphatase), ^{22}P , ^{35}S , biotin or digoxigenin. Analytically, the amplified DNA is usually electrophoresed in gels (agarose or polyacrylamide) and detected by staining with ethidium bromide (EtBr), or by hybridization, using one of the above mentioned probes, after Southern blotting (Borkowsky *et al.*, 1989; Brandt *et al.*, 1992).

2.4.3 Applications

The polymerase chain reaction provides a clinical utility for: (i) identifying HIV proviral sequences in infected individuals prior to the generation of antibodies (seroconversion) in the so called "window period", (ii) resolving the infection status of individuals with ambiguous or indeterminate serological status WB, (iii) documenting infection in seropositive individuals that test negative by other direct detection assays, (iv) demonstrating a dual HIV-I and HIV-II infection in a single individual, (v) differentiating latent HIV infection (proviral DNA) from active transcription (RNA) and (vi) monitoring the effect of antiviral therapy by determining the number of proviral copies per given number of cells thus quantifying the virus burden (Rayfield *et al.*, 1988; Wages *et al.*, 1991; Imagawa *et al.*, 1989; Ou *et al.*, 1988; Holodniy *et al.*, 1991).

An alternative to the detection of HIV proviral DNA or transcripts is the direct identification of genomic HIV RNA from intact virions. The detection

of genomic RNA also offers the opportunity to monitor viraemia in HIV patients. Ottman and co-workers (1991) have described a method for direct detection of HIV genomic RNA based on ultracentrifugation of plasma or serum samples, followed by DNase treatment of the pellet of virions. A cDNA strand is then created using an exogenous reverse transcriptase. The PCR reaction is initiated only after synthesis of cDNA has been accomplished, and the reverse transcriptase is inactivated by heating.

2.4.3.1 Diagnosis in infants

The PCR technique is particularly useful as a research tool for the diagnosis of HIV infection in babies under 18 months of age, where serological tests are of limited value due to the persistence of maternal antibodies (see Section 1, Chapter 2.1). PCR avoids this problem because it detects the presence of viral nucleic acid. Chadwick and his co-workers demonstrated the usefulness of PCR in identifying infected children as young as 2 months of age (Chadwick *et al.*, 1989). Additional advantages of PCR for infant diagnosis are that, only 500µl to 1ml of blood is required (an amount that is feasible to obtain from a newborn) and, unlike virus culture which can take up to 4 weeks or longer to complete, PCR can be done in 1 - 3 days. Borkowsky and co-workers (1992) evaluated three possible techniques for the diagnosis of HIV in infants younger than 6 months of age and concluded that PCR was just as sensitive as culture (94% vs 96%). Both these assays could detect infection in 70% to 90% of children by 2 months of life and in >90% of infants thereafter, whereas the p24 antigen assay was shown to be less sensitive.

Less information is available concerning testing for HIV-I RNA by PCR in infants and children. Escaich and co-workers used this technique to test 12 newborns within 2 weeks after birth and identified 4 infants (33%) who

were HIV-I RNA positive, compared with 7 (58%) who were HIV DNA positive by PCR (Escaich *et al.*, 1991). Although RNA PCR was less sensitive than DNA PCR, the RNA results were more predicative of which infants would rapidly progress to clinical disease. A positive HIV RNA PCR assay implies active viral replication. Samples other than blood (e.g. lymphoid tissue) may also be used to detect HIV-I RNA by PCR.

The greatest problem facing the diagnostic application of PCR (and other nucleic acid amplification methods) is false positivity due to contaminating nucleic acids (Kwok and Higuchi, 1989; Rogers *et al.*, 1991; Borskowsky *et al.*, 1992 and Petru *et al.*, 1992). The predominant source of contamination appears to be the PCR product from previous reactions (carryover) which remain because of the large number of copies generated. When numerous amplifications are performed using the same primer pair, considerable care must be taken to avoid carryover of even minute amounts of DNA from one reaction to another. A series of recommendations which should be followed in order to reduce the frequency and amount of carryover are given in Chapter 3, section 3.1.

2.4.4 Nucleic acid sequence-based amplification

A new amplification procedure termed nucleic acid sequence based amplification (NASBA) has recently been developed (Kiewits *et al.*, 1991). This nucleic acid amplification technique is based on the simultaneous activity of 3 enzymes without the necessity for thermal cycling or the intermediate addition of enzymes. This technique can be used to amplify either RNA or DNA from various sample preparations. The three enzymes are a reverse transcriptase, T7 RNA polymerase and RNase H. Bruisten and co-workers compared NASBA with reverse transcriptase PCR (RT-

PCR) for the specific detection of HIV-I RNA. Both techniques were shown to detect HIV-I viral sequences with equal efficiency (Bruisten, 1993). NASBA displayed some advantages over RT-PCR which include (i) no requirement for a separate reverse transcriptase step (ii) no necessity for a thermocycler apparatus, because the reaction is performed isothermally, and (iii) a shorter period of time for completion of the test. This makes NASBA a practical alternative for RT-PCR to detect RNA specifically.

SECTION 2

Implementation and Application of PCR to HIV-1 Investigations

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Chapter 3

Optimization of HIV-I PCR in the virology laboratory

3.1 Introduction

3.1.1 The “Standard” PCR reaction

The polymerase chain reaction has a wide variety of applications, and it is not possible to describe a single set of conditions that will guarantee success in all situations. Nevertheless, the “standard” reaction will amplify most target sequences. This reaction defines a common starting point from which changes can be attempted (Saiki, 1989; Innis and Gelfand, 1990).

The “standard” reaction is set up as a 100µl volume consisting of PCR buffer (50mM KCl, 10mM Tris.HCl, pH8.4, 1.5mM MgCl₂, 100µg/ml gelatine), 0.25µM of each primer, 200µM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP), 2.5 units of *Taq* DNA polymerase, and template DNA (10² to 10⁵ copies). A drop of mineral oil is also added, in order to prevent evaporation. However, the type of thermocycler will determine whether or not it will be necessary to add the oil, e.g. a thermocycler where the lid of the reaction tube is heated, will not require any oil overlay. The mineral oil also helps to prevent cross-contamination as it seals the reaction. Thirty-five cycles of amplification can then be performed in a DNA Thermal Cycler using the following temperature profile: Denaturation for 20sec at 94°C, primer annealing for

20sec at 55°C and primer extension for 30sec at 72°C. Cycling should conclude with a final extension step of 72°C for 5min (Saiki, 1989).

A variety of parameters can then be modified, if better results are required or if the reaction has failed. These parameters can affect the specificity, fidelity and yield of the reaction and include the following: (1) enzyme concentration (2) primer concentration (3) annealing temperature (4) dNTP concentration (5) MgCl₂ concentration in the PCR buffer, and (6) PCR buffer composition.

3.1.2 PCR Parameters

3.1.2.1 *Taq* DNA polymerase concentration

The amount of polymerase is one of the more important parameters to be optimized for a particular assay. The optimum amount of enzyme, for most assays, will be between 0.5 and 2.5 units in a 50µl-100µl reaction volume. Increased enzyme concentrations sometimes lead to decreased specificity with accumulation of non-specific background products. If the concentration is too low, an insufficient amount of desired product is made (Saiki, 1989; Innis and Gelfand, 1990).

The *Taq* DNA polymerase lacks 3'-5' proofreading activity and the fidelity (i.e. nucleotide misincorporation frequency) can depend upon the concentration of free Mg²⁺ and dNTPs, (Tindall and Kunkel, 1988; Saiki, 1989). The half-life of *Taq* DNA polymerase is 30min at 95°C, and it is recommended that no more than 30 amplification cycles should be performed (Rybicki, 1992).

There are several other thermostable DNA polymerases available on the market. The *Tfi* DNA polymerase is derived from the thermophilic

bacterium *Thermus flavus* (Rao *et al.*, 1992). This polymerase is suitable for high-temperature synthesis of DNA, with optimal activity of above 65°C, and can be used at temperatures up to 95°C. *Pfu* DNA polymerase is derived from the hyperthermophilic archaeobacterium, *Pyrococcus furiosus*. Its proofreading activity enhances the fidelity of PCR by reducing the error-rate 12-fold compared to *Taq* DNA polymerase (Lundberg, *et al.*, 1991). When *Pfu* DNA polymerase is used in PCR, it also leaves the PCR products with blunt ends, which makes it more suitable when cloning into blunt-ended vectors. Another DNA polymerase is *Tli* DNA polymerase, derived from the bacterium *Thermococcus litoralis*, which is an extremely thermostable enzyme that replicates DNA at 75°C and remains functional even after incubation at 100°C (Mattila *et al.*, 1991). *Vent* DNA polymerase is also a thermostable polymerase from *Thermococcus litoralis*, but with an added 3'-5' exonuclease activity (proofreading). This polymerase also has a function which degrades the 3'-terminal mismatches of PCR primers, thus enhancing the DNA replication fidelity (De Noronha and Mullis, 1992). The *Tth* DNA polymerase is isolated from the bacterium *Thermus thermophilus*. This enzyme also has thermostable reverse transcriptase activity that is useful in synthesizing cDNA from RNA templates (Garballeira *et al.*, 1990).

3.1.2.2 Deoxynucleotide triphosphate (dNTP) concentration

The concentration of the deoxynucleotides (dNTP) in the PCR reaction can vary from 50 to 200µM. In order to minimize misincorporation errors, the four dNTPs (dATP, dGTP, dCTP, dTTP) should be used at equivalent concentrations. The fidelity of the *Taq* DNA polymerase will be reduced if the dNTPs are imbalanced (Saiki, 1989). It is also important to remember that the dNTPs quantitatively bind Mg^{2+} , thus reducing the level of free

magnesium ions which could interfere with the polymerase activity. If any significant changes were to be made to the dNTP concentration, it would probably be necessary to change the MgCl_2 concentration in the PCR buffer as well (Innis and Gelfand, 1990; Saiki, 1989).

3.1.2.3 Magnesium concentration

The magnesium ion concentration can have an effect on the yield of the PCR products and the specificity of the reaction. Optimizing the Mg^{2+} concentration may prove to be very beneficial. Concentrations in the range of 0,5mM to 10mM are usually optimal.

3.1.2.4 PCR buffer composition

Some PCR protocols include 10% dimethyl sulfoxide (DMSO) in the standard buffer to reduce the secondary folding of the target DNA, but various reports have also shown that 10% DMSO can be slightly inhibitory to *Taq* DNA polymerase and decrease the overall yield of the amplification product (Saiki, 1989).

Gelatin or bovine serum albumin (100 $\mu\text{g}/\text{ml}$) and non-ionic detergents, such as 0,01% Tween 20 or 0,01% NP40, are essential in stabilizing the *Taq* enzyme in order to obtain maximum efficacy of the enzyme (Innis and Gelfand, 1990). The melting temperature (T_m) of the DNA products is also lowered by these additives. Sarkar *et al.* (1990) reported that the addition of formamide to the PCR buffer (1,25-2,5%) can also improve the specificity of the reaction.

3.1.3 Primer selection

In most applications it is the primer sequence and primer length that will determine the overall success of the PCR reaction. An increase in the primer length as well as an increase in the G+C content of the primer will result in an increased T_m . Consequently, 18- to 30-mer primers with a G+C content of 50-60% are generally used. The rule-of-thumb calculation: $T_m = 4(G+C) + 2(A+T)^{\circ}C$ can be applied for the calculation of an estimated T_m . It is advised that the annealing temperature (T_a) should be 5°C below the lowest T_m of the pair of primers to be used (Innis and Gelfand, 1990). It might also be useful to use the more accurate calculation (Sambrook, 1989) which takes the salt concentration into account. This calculation of the T_m will reflect the reaction conditions when the following formula is used:

$$T_m = 81,5 + 16,6[\text{Log}M + 0,41(\%G+C) - 500/L] - 0,61(\%FA),$$

where **M**= molarity of monovalent cations, usually Na^+ concentration, **L**= length of oligonucleotide in basepairs, **FA**= %formamide in the hybridization solution, and %G+C= percentage G and C nucleotides in the DNA. Innis and Gelfand (1990) also describe a few simple rules in their book to aid in the design of efficient primers.

Degenerate primers can also be used, especially when searching for new or uncharacterized sequences that are related to a known family of genes. Degenerate primers are a set of primers which have a number of options at several positions in the sequence, to allow annealing and amplification of a variety of related sequences. Degeneracies at the 3'-ends of the primers should be avoided since that could lead to inefficient extension because of mismatched bases (Rybicki, 1992; Innis and Gelfand, 1990; Saiki, 1989). An example of degenerate primers are those described in chapter 6 to amplify a subset of the *env* region (V3-region).

3.1.4 Contamination problems

The amplification that makes PCR so useful also makes it very susceptible to contamination problems. Different sources of contaminating DNA have to be considered: (1) cross-contamination between samples, resulting in transfer of target DNA from one sample to another, (2) plasmid contamination from the laboratory environment [recombinant plasmids containing cloned target sequences], and (3) carry-over contamination of amplified target DNA [amplicons]. However, the major source of concern is carry-over contamination of amplicons, due to their relative abundance and ideal structure for re-amplification (Kwok and Higuchi, 1989). Large numbers of molecules are generated in a standard reaction and each tube may contain as many as 10^{12} copies of an amplicon. Contamination of reagents, buffers, laboratory glassware, autoclaves and ventilation systems can occur as a result of this amplicon build up. In diagnostic laboratories, where assays are generally tuned for maximum sensitivity (1 to 10 template molecules), amplicon contamination can become an acute problem (Kwok and Higuchi, 1989). Amplicon contamination of reagents, pipetting devices, laboratory surfaces or even the skin of workers can yield false positive results (Kitchin *et al.*, 1990). One way to control contamination is to prevent the physical transfer of DNA between amplified samples and between positive and negative experimental controls.

The following guidelines should be applied routinely to prevent or reduce false positive rates in the laboratory:

3.1.4.1 Laboratory design and technique

It is important to physically separate the three main stages of the PCR protocol: (1) reagent preparation, (2) sample preparation and addition,

and (3) post-PCR analysis. In an ideal laboratory each of these areas should be a separate room but, as most laboratories have limited space, this separation can be achieved by providing separate dedicated workstations in two rooms. Each area should have its own dedicated equipment (pipettes, gloves, laboratory coats, tips, tubes, microfuges, waste bins, and even writing material) and these must not be transferred between the areas. By restricting access to these areas to trained personnel only, one not only reduces the distractions caused by the presence of unnecessary personnel, but also reduces potential additional sources of contamination, and the areas remain tidy and fit for their intended purpose (Kwok and Higuchi, 1989; Kwok, 1991). It is highly recommended that benchtop hoods (with UV lights for decontamination) should be used when preparing the reagents and samples, and when adding the samples. Personnel should never be allowed to enter the "clean" preparation areas after they have worked in the post-PCR areas unless they have changed their laboratory coats and gloves. The risk of contamination is greatly reduced by wearing gloves and changing them frequently. It is also advisable to use tubes with caps that do not require a great force to remove, as liquid at the bottom of the tube may splash out, causing aerosols. It is recommended that fluids be spun down to the bottom of the tube before it is opened.

3.1.4.2 Autoclaving solutions

Autoclaving can be used to sterilize those solutions and equipment that can be autoclaved without affecting their performance, but care should be exercised, especially when autoclaving pipette tips and glassware, as the autoclave can be another potential source of contamination. It is worth considering using disposable plastic-ware, pipettes and sterile commercial plugged tips.

The source of water should also be carefully considered as this could be a potentially disastrous source of contamination. It is preferable to buy water from an outside supplier or, if this is not possible, to use water from a source entirely separate from one's normal work areas.

3.1.4.3 Reagent aliquots

It is necessary to minimize the number of repeated samplings from a stock reagent. All PCR reagents can be combined into a "master-mixture" before dispensing them into a suitable volume for a single PCR reaction. The mastermixes should be checked for possible loss of activity (*Taq* enzyme) after a large batch has been prepared. Aliquots of all reagents should be made as this will also minimize the chances for sporadic contamination.

3.1.4.4 Plugged tips or positive displacement pipettes

Aerosols containing sample DNA can contaminate the barrel of pipetting devices which can lead to cross contamination of samples. This can be prevented by using disposable, plugged tips. The use of disposable plungers is also recommended when positive displacement pipettes are used in the laboratory.

3.1.4.5 Preparation and addition of template

The method used for template preparation should be kept as simple as possible, with the minimum amount of steps, to avoid creating possible contamination opportunities. In addition, the DNA sample should also be added last to minimize the opportunity for cross contamination. It is also

recommended that the specimens are prepared in a biological safety cabinet and that any clinical specimen should be treated with universal safety precautions. The work surface of such a cabinet should routinely be decontaminated with a 10% bleach solution, followed by a 70% ethanol rinse. The bleach should be made up fresh every day.

3.1.4.6 Careful choice of positive and negative controls

A highly concentrated solution of plasmid DNA, containing the target sequence, should not be used as the positive control as this can result in the introduction of many amplifiable molecules into the sample preparation area. The plasmid DNA should be diluted and, depending upon the detection system, as few as 10 to 100 copies may be sufficient to use as a positive control. The inclusion of multiple negative controls is an essential part of PCR. Firstly, a "no DNA" reagent control is included which contains all the necessary components for PCR, except the template DNA. Secondly, negative sample controls should also be included with each set of amplifications. The negative sample controls should not contain target sequences, but should be processed through all the sample preparation steps. The DNA from low risk individuals, such as laboratory personnel, can be used as the negative DNA control.

3.1.4.7 Direct methods of contamination control

The adoption of the guidelines for laboratory lay-out and good laboratory practice (described above) will serve as good preventative measures to reduce the occurrence of contamination. However, several other procedures may be introduced that can directly reduce existing contamination levels.

3.1.4.7.1 Ultraviolet light irradiation

Ultraviolet (UV) light is capable of damaging DNA and rendering it incapable of amplification by PCR (Sarkar and Sommer, 1990). The sensitivity to UV light varies enormously, as both the sequence and the size of the contaminating fragment plays a role here. Sarkar and Sommer (1990) showed in their study that DNA fragments of <700bp are highly susceptible to UV inactivation, in contrast to fragments <250bp that are much less susceptible. Irradiation of the entire area used for the pre-PCR procedures can assist in preventing “carry-over”.

3.1.4.7.2 Photochemical method

Contamination can be minimized by the addition of 4'-aminoethyl-4,5'-dimethyl-isopsoralen (4'-AMDMIP) to the PCR mixture prior to amplification (Cimino *et al.*, 1991). It does not interfere with primer annealing or *Taq* polymerase activity and is thermally stable. The tubes are exposed to longwave UV light after amplification. The UV light penetrates the tubes and photochemically activates the isopsoralen. The activated psoralen then forms a complex with pyrimidine residues on the amplified DNA that prevents *Taq* polymerase from passing along the DNA strand in the next amplification reaction. These compounds do not form interstrand crosslinks and thus do not interfere with hybridization assays when the products are being analyzed. The efficiency of this photochemical procedure can be extremely high, depending on the length and nucleotide base composition of the amplicon, and amplicons greater than 300 basepairs (bp) in length with roughly 50% G+C content can be rendered virtually completely inadequate for re-amplification. A drawback of this method is that inhibition of PCR has been observed at high isopsoralen concentrations, e.g. very short and high GC-rich amplicons need high concentrations of isopsoralen (Cimino *et al.*, 1991; Isaacs *et al.*, 1991).

3.1.4.7.3 Enzymatic method

In the enzymatic method the DNA is modified by using dUTP instead of dTTP during PCR, resulting in incorporation of dU instead of dT in the amplicon. This modified DNA does not occur naturally and can be distinguished from target DNA by the presence of this “unnatural” nucleotide base U. The bacterial enzyme, uracil-N-glycosylase (UNG) is added to the PCR reaction and digests the carry-over fragments by cleaving the uracil base from the phosphodiester backbone of any uracil-containing DNA (Fig. 9). UNG has no effect on normal thymine-containing template DNA. The enzyme is added prior to amplification so that contaminating dU-fragments are degraded and cannot serve as substrates for further amplification. The UNG is then heat-inactivated prior to PCR cycling, to prevent degradation of newly amplified uracil-containing DNA products (Longo *et al.*, 1990; Bebee *et al.*, 1992; King and Ball, 1993).

Potential problems with the UNG protocol include incomplete destruction of UNG activity when it is heat denatured before PCR cycling. As a result, residual UNG activity may affect the sensitivity of the system by inactivating uracil-containing strands as soon as they are made. It has been shown that when dUTP is substituted for dTTP it is essential to simultaneously adjust the concentrations of the dNTP and $MgCl_2$ to ensure the most efficient amplification (Perkin Elmer Cetus, 1991; Bebee *et al.*, 1992; Wang *et al.*, 1992).

When using reverse transcription PCR (RT-PCR) there is another enzymatic method, employing a restriction endonuclease digestion, that can be used to deal with contamination problems. Restriction enzymes recognize specific double-strand DNA sequences but act on the equivalent single strand sequence inefficiently, if at all. A suitable enzyme can thus selectively destroy contaminant DNA but leave the reverse

transcribed single strand cDNA intact to initiate amplification (Dougherty *et al.*, 1993).

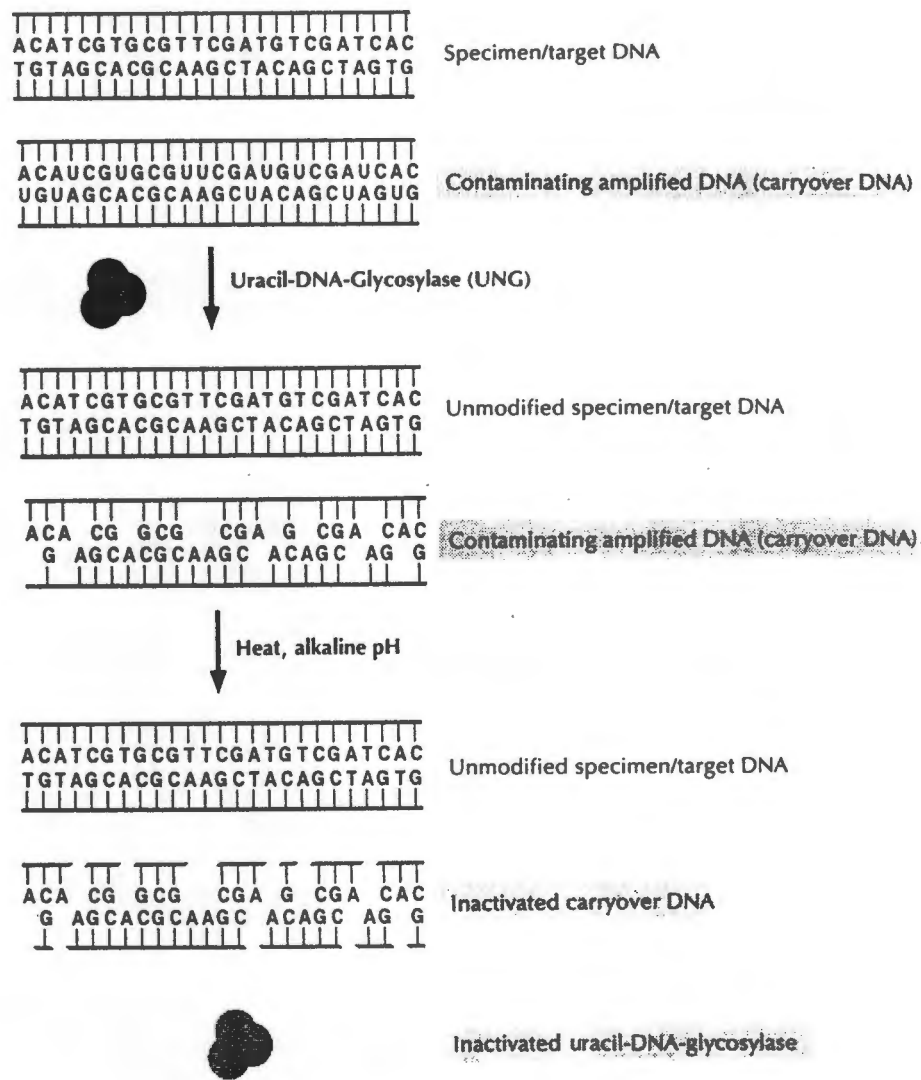


Fig. 9 Diagram of the UNG restriction-system. The top duplex molecule represents natural T-containing DNA from clinical specimens; the bottom duplex represents amplified DNA containing U. Following treatment with UNG, T-containing DNA is unaffected, but the uracil bases are cleaved from U-containing amplified DNA (Sirko and Ehrlich, 1994).

All these protocols that assist with contamination control will mostly be effective in the case of amplicon build up, and there are no quick solutions for an existing (T-containing) contamination problem. Good

laboratory practice is still highly recommended as no sterilization protocol is either 100% efficient or completely foolproof (Kwok and Higuchi, 1989).

3.2 Materials and Methods

3.2.1 Choice of primers and template

Since HIV shows marked heterogeneity, proviral sequences of HIV-I, II were amplified by using primer pairs and probes (Table 5) derived from highly conserved regions of the HIV genome. The primers and their respective probes (prepared by Professor Botes from the Department of Biochemistry, University of Cape Town) were identical in sequence to well-characterized primers and probes from published literature (Ou *et al.*, 1988; Kellogg *et al.*, 1990; Brandt *et al.*, 1992).

DNA extracted (see chapter 4.2) from lymphocytes from a known seropositive, PCR-positive adult patient was used as the template in all the PCR optimization reactions. This DNA was used in preference to a plasmid containing HIV-I sequences since that had previously led to false positive results due to amplicon contamination. Furthermore, the plasmid (pBH10) lacks the 5' LTR to which one of the primer pairs are directed.

Table 5 Different HIV1,II primers and their probes used to amplify proviral DNA isolated from peripheral blood mononuclear cells

Primer pairs and probes	Virus	Region	Sequence (5'-3')	Size of fragment	Reference
SK38 SK39 SK19	HIV-I	gag-p24	ATAATCCACCCTATCCCAGTAGGAGAAAT TTGGTCCTTGCTTATGTCAGAAATGC ATCCTGGGATTAAATAAATAGTAAGAATGTATAGCCCTAC	115bp	Ou <i>et al</i> , 1988
SK68 SK69 SK70	HIV-I	env	AGCAGCAGGAAGCACTATGG CCAGACTGTGAGTTGCAACAG ACGGTACAGGCCAGACAATTATTGTCTGGTATAGT	154bp	Ou <i>et al</i> , 1988
SK29 SK30 SK31	HIV-I	LTR	ACTAGGGAACCCACTGCT GGTCTGAGGGATCTCTA ACCAGAGTCACACAACAGACGGGCACACACTACT	104bp	Kellogg <i>et al</i> , 1990
SK145 SK431 SK102	HIV1,II	gag-p17	AGTGGGGGGACATCAAGCAGGCCATGAAAT TGCTATGTCAGTTCCCTTGTTCTCT GAGACCATCAATGAGGAAGCTGCTGCAGAAGGGAT	142bp HIV-I 139bp HIV-II	Lynch <i>et al</i> , 1992
*C1 C2	HIV-I	env	GGGTTCTTGGAGCAGCAGG GCAAATGAGTTTCCAGAGC	252bp	Ou <i>et al</i> , 1988

* Nested PCR, C1 C2 outer primer pair
SK68, 69 inner primer pair

3.2.2 Changing PCR parameters

3.2.2.1 Magnesium concentration

A dilution series of 1M MgCl_2 was made in ultrapure water and the following concentrations of 1,5mM, 2,5mM, 3,5mM, 4,5mM, 5,5mM, 6,5mM, 7,5mM, 8,5mM, 9,5mM and 10,5mM were evaluated. Unless otherwise stated, the assessment of parameters was performed using the “standard” PCR protocol (Chapter 3.1.1). Agarose gel electrophoresis and Southern blot hybridization were performed on the PCR products to evaluate the effect of different MgCl_2 concentrations, for each of the different HIV primer pairs.

3.2.2.2 *Taq* DNA polymerase concentration

A dilution series of *Taq* polymerase enzyme was made that ranged from 0.25 Units to 2.5 Units per 50 μl reaction sample. All samples contained the calculated optimum MgCl_2 concentration for the correlating primer pair. The “standard” PCR protocol was used for amplification, followed by agarose gel electrophoresis and Southern blot hybridization to evaluate the effect of the different concentrations of the enzyme.

3.2.2.3 Primer concentration

The primer concentration was not optimized. The recommended concentration of 50pmol of each primer per reaction was used after the *Taq* and MgCl_2 concentration had been optimized (Ou *et al.*, 1988; Kellogg and Kwok, 1990; Brandt *et al.*, 1992).

3.2.2.4 Annealing temperature

An annealing temperature of 55 $^{\circ}\text{C}$ was used during the amplification of all the primer pairs examined in this study as described in the literature (Ou

et al., 1988; Kellogg *et al.*, 1990; Brandt *et al.*, 1992; Lynch *et al.*, 1992).

3.2.2.5 Sensitivity check after optimization of the parameters

The sensitivity of the PCR, after optimization, was assessed by using different dilutions of the plasmid pBH10 (pUC 19 cloning vector containing an insert of a truncated HTLV-III proviral isolate of HIV-1) as template (Hahn *et al.*, 1984). The plasmid was stored as glycerol stock cultures at -70°C in the Department of Medical Microbiology at the Medical School, UCT. Plasmid DNA was amplified in bacterial cells and large scale preparations were obtained using isopycnic gradient centrifugation in caesium chloride (CsCl) gradients according to the method described by Sambrook *et al.*, (1989).

3.2.2.5.1 Plasmid preparation

A sample from the glycerol stock culture of pBH10 was streaked onto a Luria agar plate containing 1mg/ml ampicillin and incubated at 37°C overnight. A single bacterial colony was selected and inoculated into 10ml Luria broth also containing ampicillin. The culture was incubated for 6hr at 37°C with shaking, whereafter it was inoculated into 200ml Luria broth (containing ampicillin) and incubated for a further 16hr at 37°C with shaking.

Bacterial cells were harvested by centrifugation at 6000rpm at 4°C for 15min in a J2-21 Beckman centrifuge (JA-10 rotor). The culture supernatant fluid was removed and the bacterial pellet was resuspended in 3ml of 25% sucrose in 0,05M Tris-HCl pH8.0. The cell suspension was then transferred to a polypropylene tube and maintained on ice.

Cells were lysed by the addition of 10mg powdered lysozyme (Sigma) whereafter the cells were kept on ice for another 5min and 0,25M Na₂EDTA, pH8.0, was added. Cellular proteins were denatured by the addition of 5ml Triton/Doc solution (1% Triton X-100, 0,4% Na-deoxycholate in 0,01M Tris and 1mM Na₂EDTA, pH8.0). This suspension was mixed very carefully and kept on ice for 30min whereafter it was centrifuged at 15000rpm for 45min at 4°C using a Beckman JA-20 rotor to remove bacterial proteins and associated chromosomal DNA. The plasmid-containing supernatant was removed and the volume adjusted to 10ml with ultrapure water.

3.2.2.5.2 Plasmid purification

9,5g of CsCl (Boehringer Mannheim) was added to the DNA solution in order to adjust the density to 1,6g/ml. Ethidium bromide (Merck) was then added (0,2g/ml) and the solution was transferred to two polyallomer tubes, which were then heat sealed. This solution was ultracentrifuged at 50 000rpm for 20hr at 20°C in a vertical Vti65 rotor and Beckman L7-55 ultracentrifuge to separate plasmid DNA from residual cellular components. The fraction containing the plasmid was identified as a visible band upon illumination of the tubes with low energy (302nm) UV light. The plasmid band was removed from the tube by puncturing the side of the tube with a needle and syringe, and transferring the plasmid fraction into an Eppendorf microfuge tube.

Ethidium bromide (EtBr) was extracted by mixing the plasmid DNA solution with an equal volume of isoamyl alcohol, allowing the two phases to separate and discarding the upper organic phase (which should be pink as it contains EtBr). This was repeated four times. The aqueous phase was then diluted by the addition of three volumes of ultra-pure water. The plasmid DNA was precipitated by the addition of 2,5 times the volume of 100% cold ethanol at -70°C overnight. The precipitate was

concentrated by centrifugation at 12000rpm in a Beckman microfuge and the pellet was washed in 70% ethanol, dried in a Speed Vac vacuum drier and resuspended in 100µl ultra-pure water.

The yield and quality of the plasmid DNA was determined by measuring the optical density of the solution in a Beckman DU-24 spectrophotometer over a wavelength range of 220nm to 310nm. The approximate concentration of the DNA in solution could then be calculated by multiplying the absorbance of the solution by the dilution factor and by 50 (as it is known that an absorbance reading of 1 at 260nm corresponds to a DNA concentration of 50µg/ml). The yield was in the order of 65µg/ml. An estimation of the purity of the DNA was also obtained by calculating the ratio of the absorbance readings at 260nm and 280nm. A ratio of approximately 1,8 is characteristic of a pure preparation (Sambrook *et al.*, 1989).

3.2.3 Incorporation of uracil-N-glycosylase

3.2.3.1 Magnesium concentration

It was necessary to determine the optimal MgCl₂ concentration to achieve maximum amplification and to verify the suitability of the dU-containing products for further analysis. A dilution series of 1M MgCl₂ was made in ultra-pure water and concentrations ranging from 1,5mM to 10,5mM were evaluated using agarose gel electrophoresis and Southern blot hybridization after PCR was performed.

3.2.3.2 Deoxy-uridine triphosphate concentration

Substitution of dTTP with dUTP in conjunction with the use of UNG (Chapter 3.1.4.7.3) had been demonstrated to selectively prevent carry-over contamination. The optimum dUTP concentration for each primer pair was established by preparing a dilution series of dUTP (200 μ M to 1000 μ M) and including a different concentration in each separate PCR mixture. The "Standard" PCR protocol was used, followed by agarose gel electrophoresis and Southern blot hybridization, and the optimum dUTP concentration was judged by the intensities of the bands.

3.2.3.3 Incorporation of UNG

After optimizing the MgCl₂ and dUTP concentrations, the following protocol was followed to incorporate UNG (Gibco/BRL) into the PCR mixture as a preventative measure for carry-over-contamination. Half a unit UNG was added to a 50 μ l PCR reaction mixture containing the DNA template and incubated at 37^oC for 10min (Package insert, UNG Gibco BRL). The enzyme was then denatured for 10min at 94^oC before PCR was performed. The products were analysed by agarose gel electrophoresis and Southern blot hybridization.

3.2.4 PCR product manipulation

3.2.4.1 Agarose gel electrophoresis of PCR products

The PCR products were resolved by horizontal, submerged gel electrophoresis (Sambrook *et al.*; 1989) in 4,5% agarose (Promega) at 70

volts for 2hr using a TAE-buffer (0,04M Tris-acetate, 1mM EDTA pH8.0). After staining with ethidium bromide (Merck) the DNA fragments were visualised by transillumination of the gel with ultraviolet light (UV) at 302nm on a UVP transilluminator.

3.2.4.2 Southern transfer of DNA fragments

Following gel electrophoresis, the DNA fragments were transferred onto Hybond N membrane (Amersham) using a modification of the method described by Southern, 1975. The gel was rinsed in distilled water and the DNA fragments within the gel were depurinated by immersing the gel in denaturing buffer (1,5M NaCl and 0,5M NaOH) for 30min at room temperature with shaking. Hereafter, the gel was rinsed in distilled water and placed in neutralizing buffer (20mM NaOH and 1M ammonium acetate) for 40min at room temperature with shaking. A capillary blot was set up by placing the gel onto a clean glass plate, wetting the nylon membrane (Hybond N) with neutralizing buffer and placing it on top of the gel. A plastic disposable pipette was used to roll out any air bubbles that might have been trapped under the membrane and which might prevent complete transfer of the DNA. Three sheets of 3MM (Whatman) paper were cut to size, soaked in neutralizing buffer and placed on top of the Hybond N membrane. The 3MM paper was, in turn, covered with a generous amount of paper towels and weighted with a glass plate and bricks. Transfer was allowed to proceed overnight. After careful dismantling of the apparatus, the membrane was marked with pencil to allow later identification of the lanes. The DNA was cross-linked to the membrane by exposure of the membrane to short wave UV light (250nm) in a UV-crosslinker (Hoefer Scientific Instruments). The membrane was then briefly washed in 6X SSC (sodium chloride /sodium citrate buffer) before hybridization.

3.2.4.3 Digoxigenin labelling of oligonucleotides

Oligonucleotide probes were labelled with digoxigenin-dUTP (DIG-dUTP) using a DIG oligonucleotide tailing kit (Boehringer Mannheim) to generate the non-radioactive probe. For each reaction, oligonucleotides corresponding to 100pmol 3'OH ends (5µl) were mixed with 4µl of 5X tailing buffer (1M potassium cacodylate; 0,125M Tris.HCl; 1,25 mg/ml bovine serum albumin, pH6.6), 4µl of 25mM CoCl₂; 1µl of 1mM DIG-dUTP; 1µl of 10mM dATP; 1µl of terminal transferase, and made up to a final volume of 20µl with ultra-pure water. The reaction was incubated at 37°C for 3hr and placed on ice. Two microlitres of a glycogen-EDTA solution (1µl 20mg/ml glycogen, and 200µl 0.2M EDTA, pH8.0) was added to stop the reaction. The labelled oligonucleotide was then precipitated with 2,5µl 4M LiCl and 75µl prechilled (-20°C) absolute ethanol and left at -70°C for at least 90min, before centrifugation at 12000g. The pellet was washed with 50µl of cold 70% (v/v) ethanol, dried under vacuum and dissolved in 10µl of ultra-pure water. The labelled probe (10µl) was then added to 20ml of prehybridization solution (6X SSC, 1% blocking solution [Boehringer Mannheim] and 0,1% SDS), to make it ready for use. The DIG-system guarantees the detection of 0,03pg homologous DNA using chemiluminescence (Boehringer Mannheim, 1993).

3.2.4.4 Hybridization of labelled oligonucleotides to DNA fragments

Membranes bearing immobilized HIV-1 DNA fragments were incubated at 42°C for 4hr, using a shaking water bath, in an aqueous prehybridization solution (see above). A formamide hybridization solution is usually recommended when a RNA probe is used in the hybridization step. The prehybridization solution was then replaced with hybridization solution

containing the DIG-labelled probe (chapter 3.2.4.3). Hybridization was allowed to take place overnight at 42°C in a shaking water bath. After each hybridization procedure, the membrane was washed at successively higher stringency conditions until optimal specificity was reached, as shown by chemiluminescent detection. A combination of high salt concentration in the wash buffer and low temperatures are usually referred to as a low stringency condition, whereas a combination of low salt concentration and high temperatures usually indicate a high stringency condition.

To determine optimum specificity the blot was first washed in 2X SSC; 0,1% (w/v) SDS at 42°C for 2x5min and then in 0,1X SSC; 0,1% SDS for 2x15min at increasing temperatures (thus increasing the stringency condition) ranging from room temperature to 88°C.

3.2.4.5 Chemiluminescent detection of the hybridized probes

The blots were washed briefly (1-5min) in washing buffer consisting of 0,3% Tween-20 in buffer 1 (0,1M maleic acid, 0,15M NaCl, pH7.5), and then immersed for 30min at room temperature in buffer 2 [blocking stock solution (proteolytic fragments of casein in powder form) diluted 1:10 in buffer 1]. The membranes were then covered with antibody conjugate solution (anti-digoxigenin - AP, Fab fragments) diluted 1:10 000 in buffer 2, and gently shaken for 30min at room temperature. The unbound conjugate was removed by washing the blots for 2x15min at room temperature in washing buffer. An equilibration step of 2-5min in buffer 3 (0,1M Tris-HCl, 0,1M NaCl, 50mM MgCl₂, pH9.5) preceded the addition of the diluted substrate [lumigen PPD, 4-methoxy-4-(3-phosphate-phenyl-spiro-1,2-dioxetane-3,2'-adamantane) disodium salt] (1:100 in buffer 3) before the blots were dried on 3MM (Whatman) paper and sealed in plastic bags. The sealed blots were pre-incubated for 15min at 37°C

before exposure for 90min at room temperature to X-ray (Agfa Curix RPI) film, in the presence of a Cronex intensifying screen. After exposure, the X-ray film was developed by immersion in developer for 3min, stop solution for 1min and fixer for 3min. The developer used was Polycon A (Champion photochemistry) diluted 1 in 5. The stop solution consisted of 2% (v/v) acetic acid, and the fixer used was Amfix (Champion photochemistry) diluted 1 in 4.

3.3 Results

3.3.1 Changing PCR parameters

3.3.1.1 Optimum magnesium concentration for PCR mixtures containing dTTP or dUTP

The optimum MgCl_2 concentration was determined by the most intense band of PCR product after Southern blot hybridization with a DIG-labelled oligonucleotide probe.

The chemilumigram of primer pair SK145,431 showed equally strong signals at both extremes of the dilution series, i.e. at 1,5 - 3,5mM and at 10,5mM. Weaker signals were detected in the 4,5mM to 9,5mM range (Fig. 10).

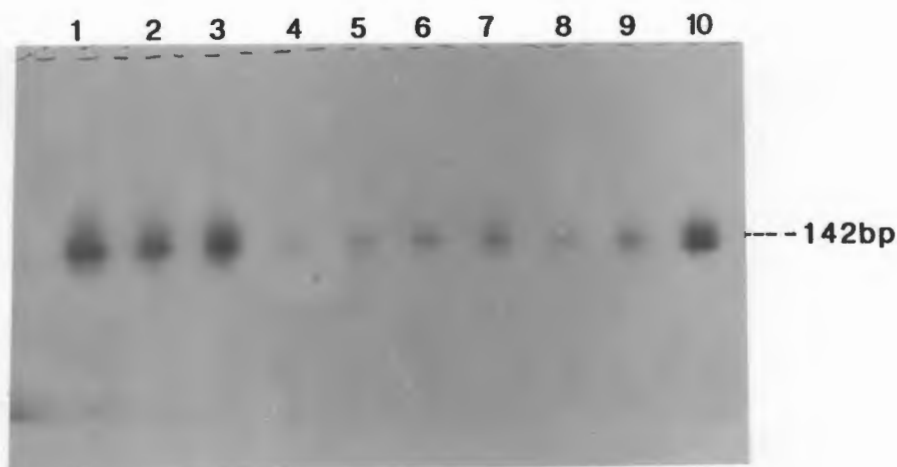


Fig. 10 Optimization of the MgCl_2 concentration for primer pair **SK145,431**, using dTTP-containing (200 μM) mastermixes, is illustrated in the magnesium titration above. Increasing MgCl_2 concentrations are shown from left to right: Lane 1 1,5mM; Lane 2 2,5mM; Lane 3 3,5mM; Lane 4 4,5mM; Lane 5 5,5mM; Lane 6 6,5mM; Lane 7 7,5mM; Lane 8 8,5mM; Lane 9 9,5mM; Lane 10 10,5mM. Ten microlitres of the PCR products was separated on a 4,5% agarose gel, the DNA was transferred to a nylon membrane by Southern blot and hybridized with a DIG-labelled oligonucleotide probe.

The primer pair SK38,39 showed a similar pattern, of two strong signals. This phenomenon was not observed for primer pair, SK29,30.

Established optimum MgCl_2 concentrations (Table 6) for the respective HIV primers when using dTTP-containing mastermixes were: 1,5mM for SK38,39; 2,5mM for SK29,30; 1,5mM for SK68,69; and 1,5mM for SK145,431. In the case of primer pairs SK145,431 and SK38,39 where equally strong signals were shown, the lower concentration of MgCl_2 was chosen for economic reasons

It was necessary to re-adjust the MgCl_2 concentration, when dUTP-containing mastermixes were used for HIV PCR. The optimum concentrations were determined in the same manner as for the dTTP mastermixes and were found to be: 3,5mM for SK38,39 (Fig. 11).

The optimum Mg^{2+} concentration for the primer pair SK29,30 was not so clear (Fig. 12), and a concentration of 3,5mM was chosen on the basis of best sensitivity and specificity (compared to the dTTP control included in the PCR run), as judged by the intensity of the band (maximum sensitivity), with minimal non-specific bands present (maximum specificity).

An optimal MgCl_2 concentration of 4,5mM was determined for SK145,431 in the dUTP system (results not shown).

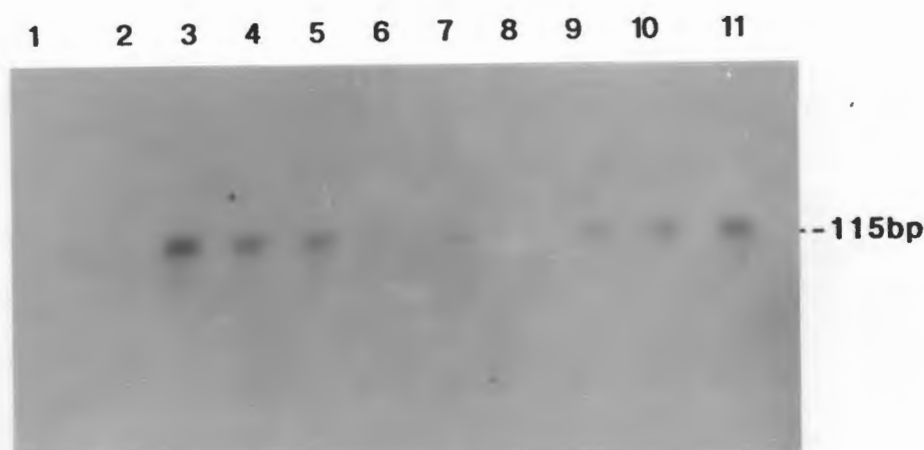


Fig. 11 Optimization of the MgCl_2 concentration for primer pair **SK38,39**, using dUTP-containing (200mM) mastermixes. Increasing MgCl_2 concentration are shown from left to right: Lane 1 1,5mM; Lane 2 2,5mM; Lane 3 3,5mM; Lane 4 4,5mM; Lane 5 5,5mM; Lane 6 6,5mM; Lane 7 7,5mM; Lane 8 8,5mM; Lane 9 9,5mM; Lane 10 10,5mM. Lane 11 is the dTTP control containing 1,5mM MgCl_2 . Ten microlitres of the PCR product was separated on a 4,5% agarose gel, the DNA was transferred to a nylon membrane by Southern blot and hybridized with a DIG-labelled oligonucleotide probe.

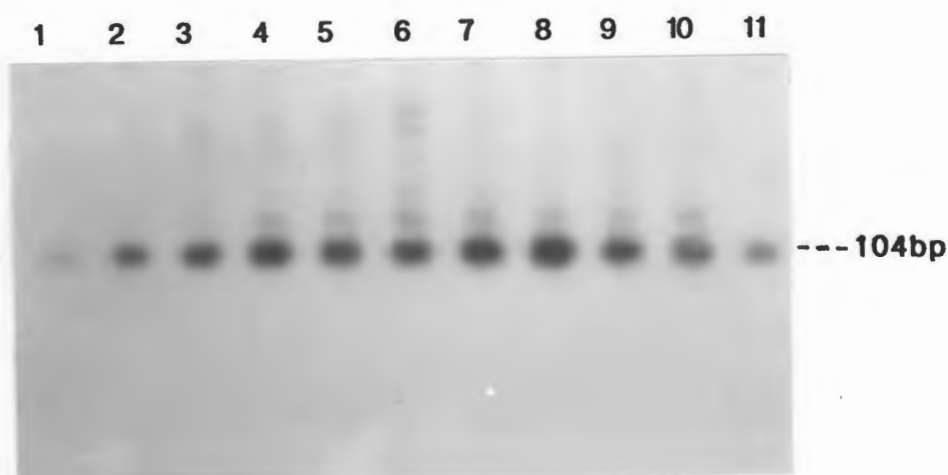


Fig. 12 Optimization of the MgCl_2 concentration for primer pair **SK29,30**, using dUTP-containing (200mM) mastermixes. Increasing MgCl_2 concentration are shown: Lane 1 1,5mM; Lane 2 2,5mM; Lane 3 3,5mM; Lane 4 4,5mM; Lane 5 5,5mM; Lane 6 6,5mM; Lane 7 7,5mM; Lane 8 8,5mM; Lane 9 9,5mM; Lane 10 10,5mM. Lane 11 is the dTTP control containing 1,5mM MgCl_2 . Ten microlitres of the PCR product was separated on a 4,5% agarose gel, the DNA was transferred to a nylon membrane by Southern blot and hybridized with a DIG-labelled oligonucleotide probe.

Table 6 Optimum magnesium concentration for the different HIV primer pairs

<i>Primer pairs</i>	<i>Optimum MgCl₂ concentration for dTTP-containing mastermixes</i>	<i>Optimum MgCl₂ concentration for dUTP-containing mastermixes</i>
SK38,39	1,5mM	3,5mM
SK29,30	2,5mM	3,5mM
Sk68,69	1,5mM	*Not done
SK145,431	1,5mM	4,5mM

*This primer set was eliminated from the study due to early contamination problems.

3.3.1.2 Optimum *Taq* DNA polymerase concentration

The optimal concentration of *Taq* DNA polymerase was determined by the intensity of the HIV-specific band on an EtBr-stained agarose gel. Two of the primer pairs, SK145,431 (Fig. 13) and SK29,30, showed an increasing accumulation of non-specific background products as the enzyme concentration was increased. This was not noted with primer pair SK38,39 (Fig. 14). With all the primer pairs it was also observed that when very low enzyme concentrations were used in the reaction, an insufficient amount of product was made (not visible on an agarose gel). For this reason it was decided to confirm positive PCR specimens by Southern blot hybridization with a DIG-labelled probe.

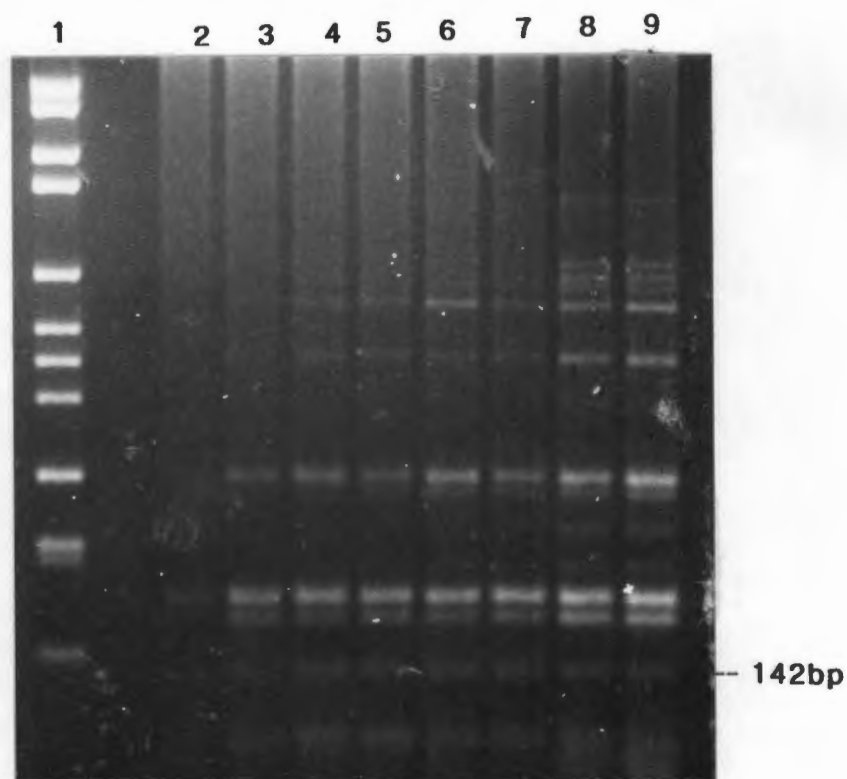


Fig. 13 Optimization of the *Taq* DNA polymerase concentration for primer pair **SK145,431**. Lane 1, MWVI; Lane 2, 0,25U; Lane 3, 0,5U; Lane 4, 0,75U; Lane 5, 1,0U; Lane 6, 1,25U; Lane 7, 1,5U; Lane 8, 2,0U; Lane 9, 2,5U. Ten microlitres of PCR product was separated on a 4,5% agarose gel, and stained with EtBr.

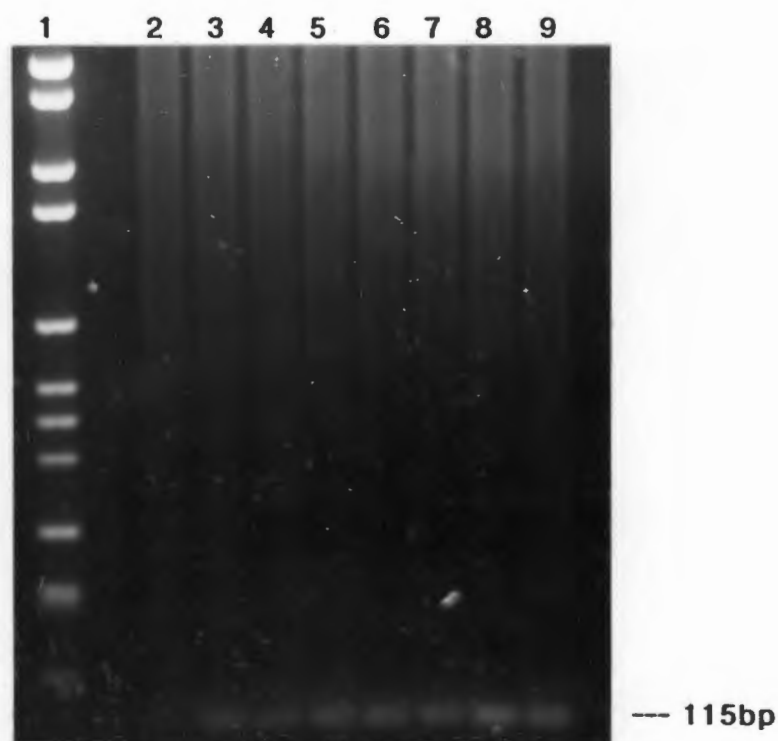


Fig. 14 Optimization of the *Taq* DNA polymerase concentration for primer pair **SK38,39**. Lane 1, MWVI; Lane 2, 0,25U; Lane 3, 0,5U; Lane 4, 0,75U; Lane 5, 1,0U; Lane 6, 1,25U; Lane 7, 1,5U; Lane 8, 2,0U; Lane 9, 2,5U. Ten microlitres of PCR product was separated on a 4,5% agarose gel, and stained with EtBr.

Chosen optimal *Taq* DNA polymerase concentrations are summarized in Table 7.

Table 7 Optimum *Taq* concentration for the different HIV primer pairs

<i>Primer pair</i>	<i>Optimum Taq concentration for HIV PCR</i>
SK38,39	1,0 units
SK29,30	1,5 units
SK68,69	1,5 units
SK145,431	1,0 units

3.3.1.3 Optimum concentration of dUTP when using the UNG-system

The substitution of dUTP required an adjustment of the dUTP concentration to obtain the same sensitivity as that shown by the dTTP system. The optimum dUTP concentrations were determined by comparing the intensity of the bands produced with different dUTP concentrations (as detected on the chemilumigram after Southern blot hybridization) with that of the control dTTP (200 μ M) band and selecting the minimal dilution that gave comparable signals. The optimal dUTP concentrations were similar for all 3 HIV primer pairs tested: 400-600 μ M for SK38,39; 400 μ M for SK29,30; and 400-600 μ M for SK145,431 (Fig. 15).

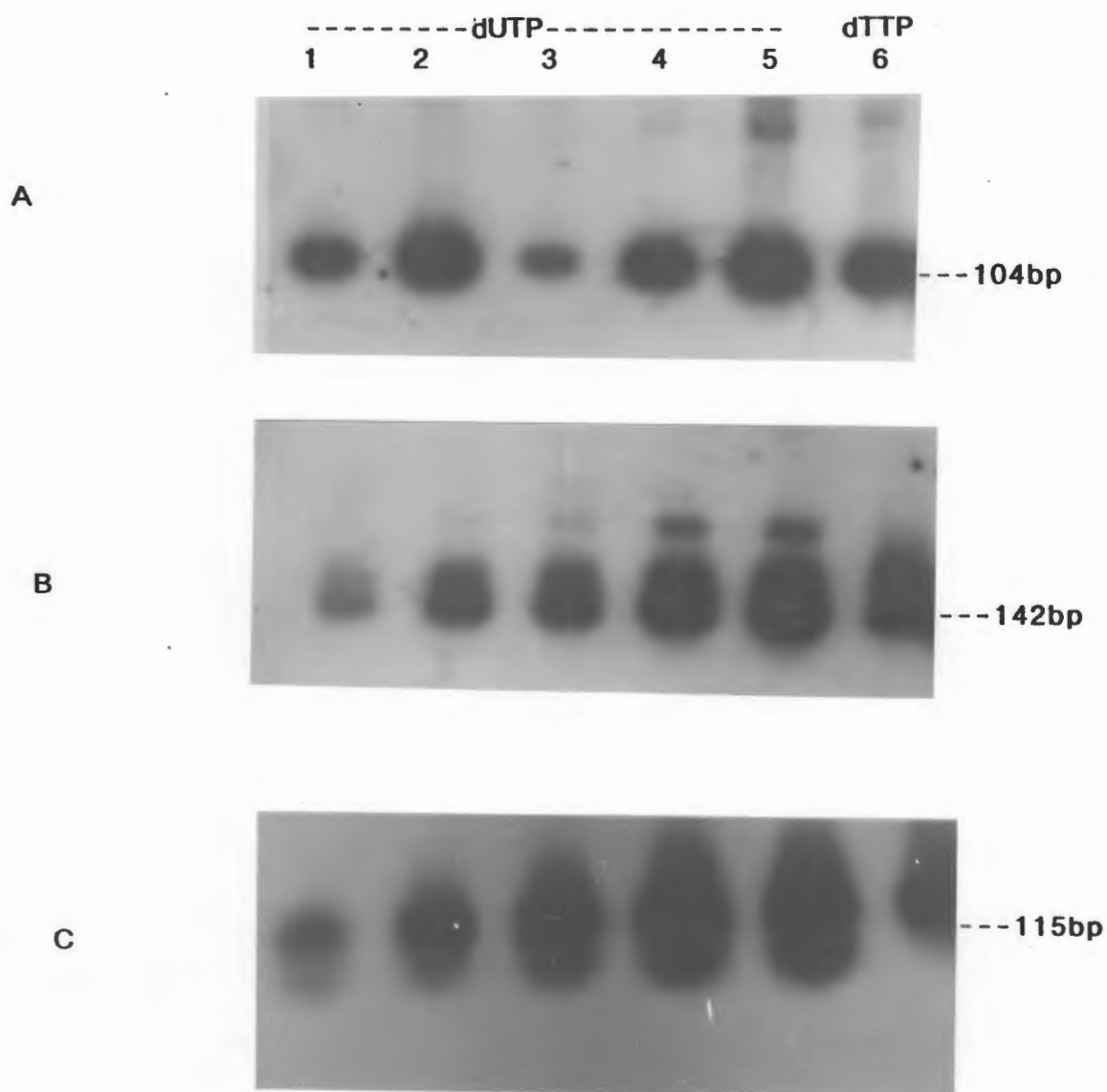


Fig. 15 Optimization of the dUTP concentration for primer pairs **SK29,30 (A)**, **SK145,431 (B)**, and **SK38,39 (C)** is illustrated above. Increasing dUTP concentrations are shown from left to right: (A , B and C): Lanes 1, 200μM; Lanes 2, 400μM; Lanes 3, 600μM; Lanes 4, 800μM; Lanes 5, 1000μM; and Lanes 6, 200μM dTTP control. Ten microlitres of the PCR product was separated on a 4,5% agarose gel, the DNA was transferred to a nylon membrane by Southern blot and hybridized with a DIG-labelled oligonucleotide probe.

Table 8 Optimum dUTP concentration compared with the dTTP concentration for the different HIV primer pairs

<i>Primer pair</i>	<i>Optimum dUTP concentration</i>	<i>dTTP concentration</i>
SK38,39	400-600 μ M	200 μ M
SK29,30	400 μ M	200 μ M
SK68,69	*Not done	200 μ M
SK145,431	400-600 μ M	200 μ M

*Due to contamination problems, this set of primers was eliminated from the study before the introduction of the UNG-system.

3.3.1.4 Confirmation of HIV PCR sensitivity after optimization of the magnesium-, *Taq*-, and dUTP concentrations

3.3.1.4.1 Confirmation of sensitivity using a dilution series of pBH10

After optimization of the MgCl₂, *Taq* DNA polymerase and dNTP concentrations, the PCR sensitivity was confirmed using different dilutions of the plasmid pBH10. The plasmid titration showed that the optimised system could detect as little as one copy of plasmid when using either of the primer pairs, SK38,39 (Fig. 16A) or SK145,431 (Fig. 16B).

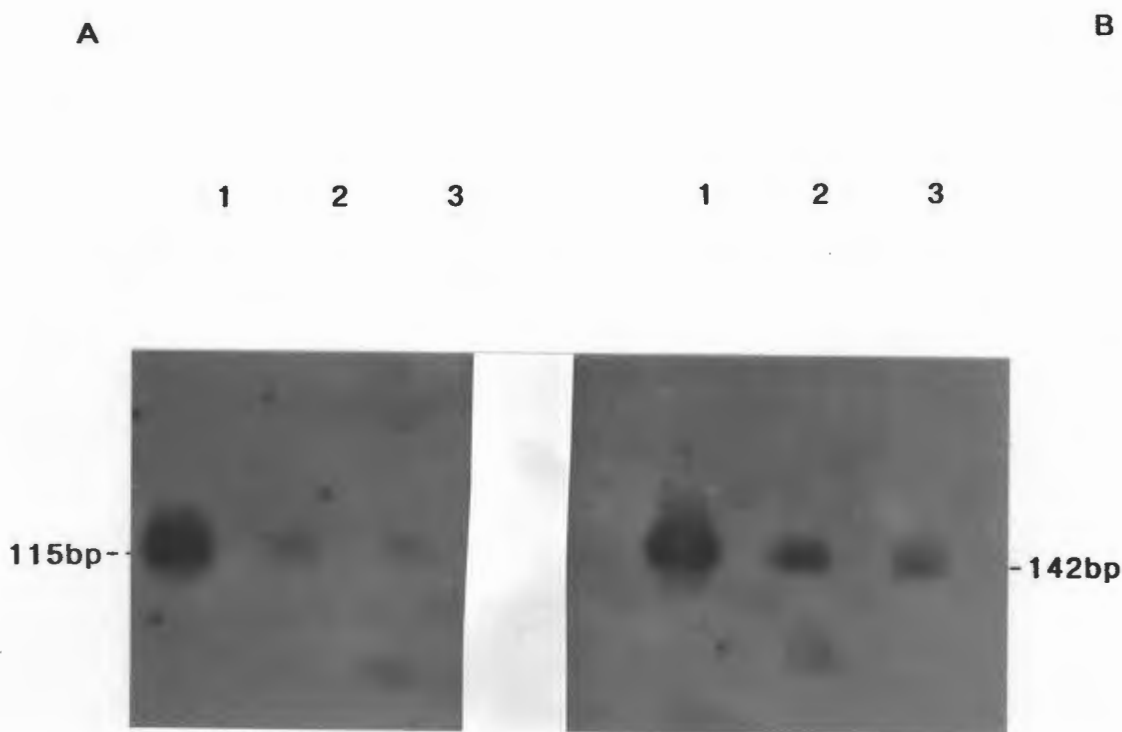


Fig. 16 Confirmation of HIV PCR sensitivity after optimization, using the primer pairs **(A) SK 38,39**, and **(B) SK145,431** with decreasing amounts of the plasmid (pBH10). Lane 1, 100 copies; Lane 2, 10 copies; and Lane 3, 1 copy. Ten microlitres of the PCR product was separated on a 4,5% agarose gel, the DNA was transferred to a nylon membrane by Southern blot and hybridized with a DIG-labelled oligonucleotide probe.

The LTR primer set was also subjected to this sensitivity test, and as expected, gave negative results (no amplification), probably because the plasmid, pBH10 only contains a fragment of one of the LTR regions of HIV and is thus not a suitable template for this primer set.

3.3.1.4.2 Comparative sensitivity of PCR systems containing either dTTP or dUTP for use on clinical material

DNA from three PCR-positive samples (2 adults and 1 infant) were used in duplicate to compare the dTTP system with the dUTP system. The two systems displayed comparable sensitivity as judged by the intensity of the DNA bands obtained after Southern blot hybridization. There was no significant loss of product yield when the dUTP system was used (Fig. 17).

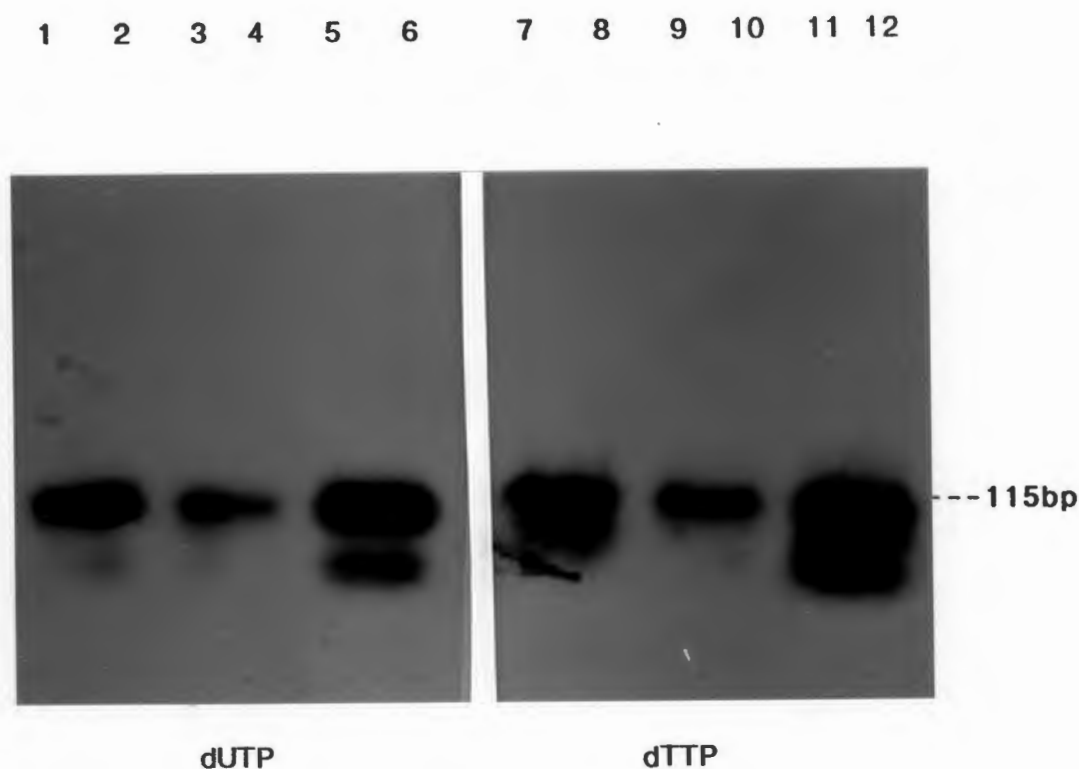


Fig. 17 A comparison of the dTTP and dUTP systems after optimization, on duplicate clinical samples: dUTP PCR (lanes 1 - 6): lanes 1 and 2, pos adult patient no.1; lanes 3 and 4, pos infant; lanes 5 and 6, pos adult patient no. 2. dTTP PCR (lanes 7 - 12): lanes 7 and 8, pos adult patient no. 1; lanes 9 and 10, pos infant; lanes 11 and 12, pos adult patient no. 2.

3.3.2 Incorporation of UNG as a preventative measure for “carry-over” contamination

The incorporation of the UNG enzyme in the reaction mixture prior to amplification resulted in a substantial loss of amplification signal after Southern blot hybridization (Fig. 18). The enzyme was therefore titrated to determine whether lowering the concentration would improve the amplification signal. In addition, the PCR protocol was altered to include an extra step at the end of the program whereby the reactions were held

at 72°C, prior to any analysis. Neither of these alterations yielded a significant improvement and a loss of product yield and sensitivity was still noted (results not shown). Likewise, storage of the PCR products at 4°C before analysis did not result in an improved detection. The adverse effect on the product yield induced by the UNG-system was not only apparent when using dUTP-containing mastermixes, but were also observed when dTTP-containing mastermixes were used (not shown).

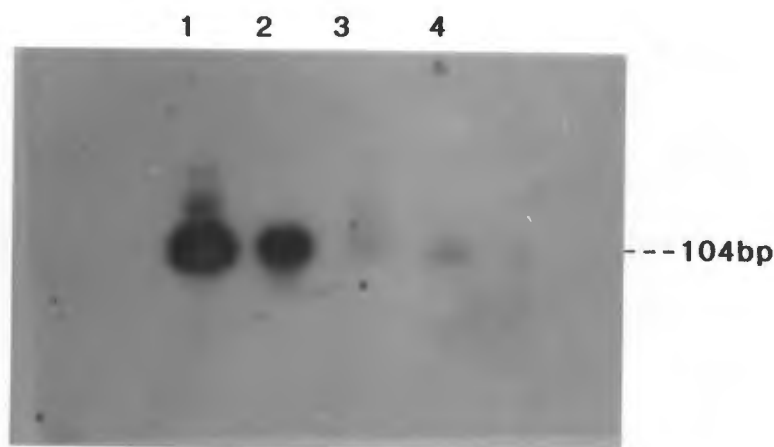


Fig. 18 The incorporation of UNG (to control "carry-over" contamination) is shown after HIV PCR and Southern blot hybridization have been performed using primer pair SK29,30. Lanes 1 and 2, positive patient DNA amplified using dUTP mastermixes **without** the addition of UNG; lanes 3 and 4, DNA from the same positive patient, but amplified using dUTP mastermixes **with** the incorporation of 0,5U UNG and heat inactivation before the onset of PCR.

3.3.3 Optimal stringency conditions when hybridizing with DIG-labelled oligonucleotide probes

Optimal specificity and sensitivity after Southern blot hybridization was achieved by using a 42°C washing temperature combined with a low salt wash buffer (0,1X SSC, 0,1% SDS). Higher temperatures, using the same low salt buffer, resulted in weaker signals on the chemilumigram,

indicating a loss of sensitivity at these higher temperatures. A faint signal was still visible after washing the membranes at 78°C, but no signal was visible when the membranes were exposed to a washing temperature of 88°C (Fig. 19).

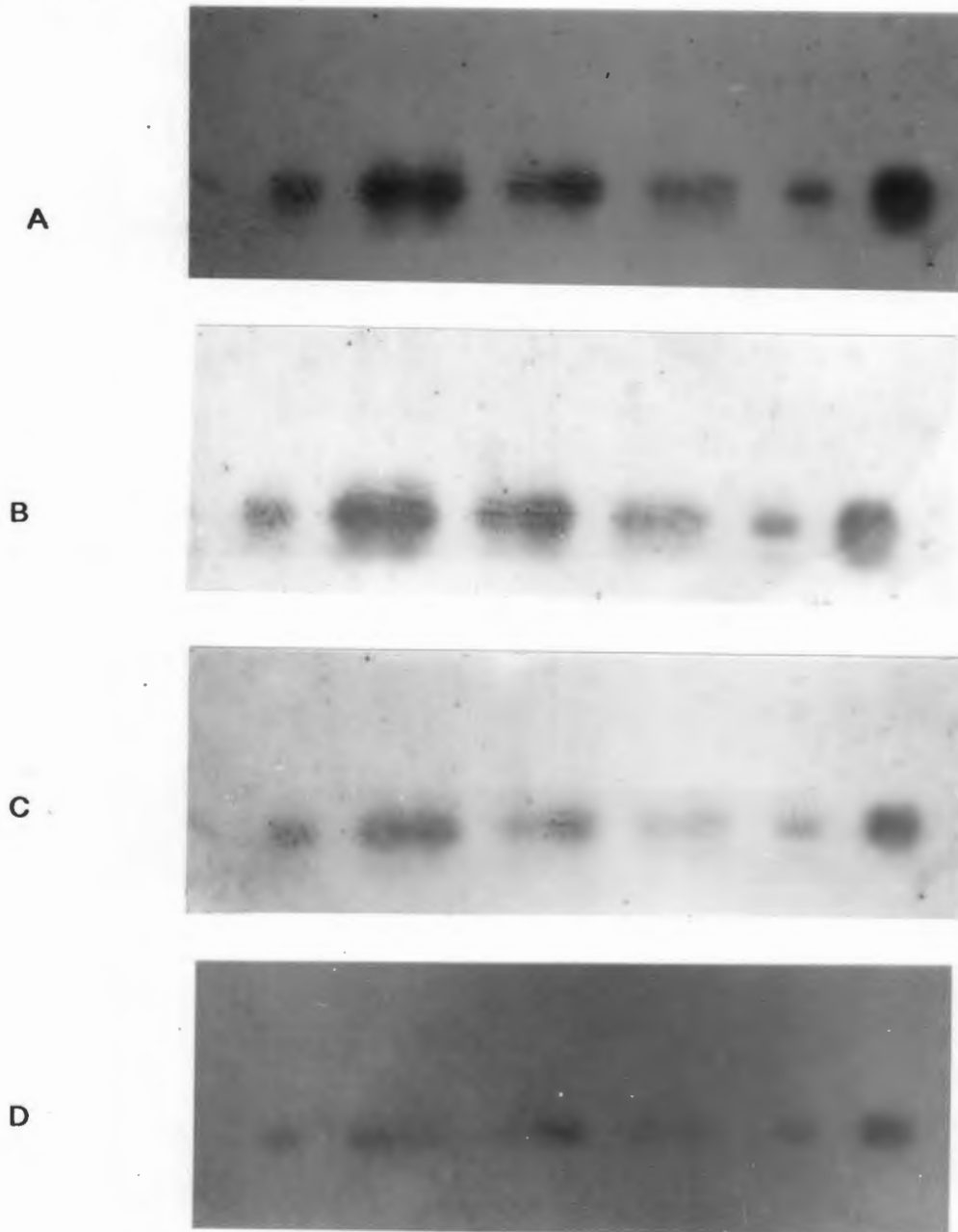


Fig. 19 Optimal stringency washing conditions were determined by increasing the temperature during the 2X15min washing step (with the 0,1X SSC, 0,1% SDS buffer) after hybridization, (A) 42°C; (B) 57°C; (C) 67°C; (D) 78°C.

Temperatures above 42°C resulted in progressive loss of signal.

3.4 Discussion

Before a laboratory assay can be applied in a diagnostic system, it is essential that it be evaluated to ensure maximum specificity and reproducibility so that false positive diagnoses are eliminated. Maximum sensitivity is also required to avoid false negative results. In the case of PCR it is not always possible to simultaneously optimize each criterion for both those samples that contain high copy numbers and those with very low copy numbers of target DNA. In this study the protocol was optimized for low copy numbers since the work was aimed primarily at the screening of infants.

It is known that both specificity and sensitivity are affected by the levels of Mg^{2+} in the PCR reaction, and these results showed that the optimal concentration of $MgCl_2$ varied depending upon the choice of dNTP (the concentration of available Mg^{2+} is affected by the phosphate concentration of the buffer, and dNTPs are the main source of phosphate groups). Concentrations of 1,5mM $MgCl_2$ and 200 μ M of dTTP were optimal when using the dTTP-system, however, when the dUTP-system was used a two-fold to three-fold increase in the $MgCl_2$ and dUTP concentrations were required to achieve similar results to those shown by the dTTP-system. The higher concentrations of dUTP that were needed probably dictates the simultaneous requirement for higher $MgCl_2$ concentrations, since high concentrations of dNTPs appear to quantitatively bind Mg^{2+} , and hence reduce the available magnesium in the PCR buffer. This in turn could also affect the efficiency of the *Taq* DNA polymerase. The observed accumulation of non-specific amplification products at higher Mg^{2+} concentrations (3,5mM to 9,5mM) corroborates observations made by Erlich (1989). In the case of 2 primer pairs (SK38,39 and SK145,431) it was not possible to distinguish between two apparently optimal levels of $MgCl_2$. The reasons for this are

not clear, but since there was little effect on the final degree of amplification the lower concentration of $MgCl_2$ was chosen, both for economic reasons and to avoid possible undue ill effects on the performance of the *Taq* enzyme (Innis and Gelfand, 1990; Saiki, 1989).

All four primer pairs performed well and the optimal *Taq* DNA polymerase concentrations for each of the primer pairs were found to be similar (1,0 to 1,5 units). The concentrations required were in some instances higher than those described by other researchers. For example, Simmonds *et al.* (1990) used 1,5U/100 μ l reaction volume, Ou *et al.* (1988) used 0,6U/50 μ l and Kwok and Sninsky (1993) used 2,5U/100 μ l in their HIV PCR reactions. In titrations with two different primer pairs, SK29,30 and SK145,431, it was noted that an increase in *Taq* concentration led to an accumulation of non-specific background products. Although this might indicate decreased specificity, it was ensured that only HIV specific products were detected by making use of specific probes. It is nonetheless, essential to use some excess of polymerase when PCR is applied as a diagnostic assay for different patient samples, because of the potential inhibiting activity of haem-related products in the sample.

After the optimal $MgCl_2$ -, *Taq*-, and dNTP concentrations had been incorporated into the standard protocol, a PCR run was performed on a dilution series of pBH10 to verify the sensitivity of the assay for each primer pair. Whether a single band, many bands or a smear was visible on the gel, Southern blot analysis and oligonucleotide hybridization was performed as a specificity control. The fact that the optimized system was able to detect a single copy of target plasmid DNA was encouraging. However, it is necessary to bear in mind that results obtained with dilute samples do not give a true reflection of the situation in diagnostic assays where clinical samples may vary widely from one another. It might in future be useful to employ a quantitative assay to determine whether the

target copy number in the patient sample is sufficient or too low,. Alimenti *et al.* (1994) described the quantitation of HIV in whole blood of infected children using a quantitative microculture method for PBMCs, and Menzo *et al.* (1992) developed and optimized a competitive RT-PCR assay for the quantitative detection of HIV-1 viremia in PBMCs. In the present study the use of a nested primer set, which can potentially increase the sensitivity of the assay, was ruled out as this type of system is very prone to "carry-over" contamination and thus increases the opportunity of false positive results. This decision was re-inforced by early experiments during which the nested *env* primer pair (C1C2 as outer primers and SK68,69 as inner primers) was eliminated from this study as a result of contamination which could not be cleared from our diagnostic system. Amplicons were detected in the buffers as well as in the water bath situated in the DNA preparation room (results not shown) requiring many months of effort and stringent measures to eliminate.

It was found that the specificity of the assay could be further increased by optimizing the stringency conditions of the washes after oligonucleotide hybridization. The combination of a temperature of 42°C, with a low salt concentration in the washing buffer (0,1X SSC) provided very stringent conditions which guaranteed the optimum specificity and sensitivity of the assay.

The greatest concern in PCR protocols is the inadvertent introduction of contamination. For that reason, dUTP-system was chosen in preference to dTTP-system (despite the fact that the two dNTPs performed equally well). This was a precautionary measure so that in the event of amplicon-related contamination it could more easily be controlled by utilizing the UNG enzyme. However, although this enzyme has been put forward as a solution to amplicon contamination problems, in our hands it was not successful as the presence of the enzyme appeared to inhibit the

amplification. The loss of amplification signal, after using the UNG-protocol, could be due to the breakdown of amplification products by residual UNG activity during or shortly after amplification. However, decreasing the enzyme concentrations did not alleviate the problem, nor did extreme changes in the temperature of the PCR product prior to gel electrophoresis have any beneficial effect. Similar observations regarding the adverse effect of UNG have been made by other researchers when using the UNG-protocol during PCR assays for amplification of mycobacterial sequences (B. Allan, personal communication). As the UNG enzyme compromised the sensitivity of the PCR protocol, it has been omitted from all routine PCR amplifications. However, dUTP is still incorporated and should any "carry-over" contamination problem arise, the enzyme can be incorporated as one of the measures used in a broad program of eradication to clear all the contaminants. The PCR protocol could then be continued using dTTP-containing mastermixes.

Chapter 4

Application of HIV PCR in amplification of HIV-1 sequences from seropositive sera.

4.1 Introduction

Serological assays are used to identify persons who have been exposed to HIV-I or -II, but they do not conclusively determine a current infection as indicated by the frequent occurrence of serology indeterminate results. The most reliable demonstration of a viral infection is based on isolation of the virus by cell culture or on the detection of the viral genome in patients' cells (Laure *et al.*, 1988). The isolation of HIV involves prolonged co-cultivation of peripheral blood mononuclear cells (PMBCs) with phytohemagglutinin (PHA)-stimulated lymphocytes from an uninfected donor. This procedure is labour intensive, time consuming, costly, requires special laboratory facilities that are not readily available in most diagnostic laboratories, and lacks sensitivity in that viruses cannot be isolated consistently from persons with documented infections (Ou *et al.*, 1988; Loche and Mach, 1988). The polymerase chain reaction can be used directly to determine the presence of HIV genetic information in the DNA from PBMCs of a patient (Laure *et al.*, 1988; Ou *et al.*, 1988; Loche and Mach, 1988; Lifson *et al.*, 1990). The PCR approach has been reported to be extremely sensitive (detecting 1-10 molecules of HIV DNA in the total nucleic acid from one million cells (Ou

et al., 1988), and allowing the detection of infection (presence of viral DNA) in the early stages before antibody production begins, sometimes referred to as the “window period” (Imagawa *et al.*, 1989).

After optimization of the PCR parameters (Chapter 3), this technique was used to detect HIV-I,II proviral sequences in PMBCs of patients who were screened in the routine laboratory for HIV antibodies. The objective was to correlate the PCR results with the serological status of these patients and thus to gauge the sensitivity and specificity of the optimized PCR protocol.

4.2 Materials and Methods

4.2.1 Patient population

The study population consisted of 153 randomly selected patients whose blood was sent for HIV screening to the routine diagnostic laboratory in the Department of Clinical Virology at Groote Schuur Hospital (Table 9). Some of the patients either had clinical signs that were suggestive of an HIV infection (such as pneumonia, tuberculosis, Kaposi's sarcoma) or they were considered to be at risk of being infected with HIV e.g. infants of HIV positive mothers or sexual contacts of HIV-infected persons. However, for a number of patients, the clinical details were not supplied on the request forms. The study population included 55 males (8 white males, 12 coloured males, and 35 black males), 57 females (2 white females, 17 coloured females, and 38 black females) and 41 whose gender was unknown.

Table 9 Source of blood samples from 153 randomly selected patients referred for HIV screening

<i>Institutions</i>	<i>Number of patients tested</i>
<i>Groote Schuur Hospital</i>	35
<i>Red Cross War Memorial Childrens Hospital</i>	26
<i>Somerset Hospital</i>	25
<i>Private Pathologists</i>	13
<i>Hottentots Holland Hospital</i>	9
<i>Paarl Hospital</i>	6
<i>George, Oudtshoorn, Mosselbay Hospitals</i>	5
<i>Mowbray Maternity Hospital</i>	4
<i>Conradie Hospital</i>	3
<i>Brooklyn Chest Hospital</i>	3
<i>Victoria Hospital</i>	2
<i>Stellenbosch Hospital</i>	1
<i>Tygerberg Hospital</i>	1
<i>*Unknown institutions</i>	20

*Unknown details due to incomplete information received on the request forms.

Serum samples were initially screened for the presence of HIV antibodies. As a follow-up, a sample of each seropositive individual's blood was collected in a tube containing ethylenediaminetetra-acetic acid (EDTA) and processed to separate the PBMCs

4.2.2 HIV antibody detection

All the serum samples were initially evaluated for antibodies to HIV-I and HIV-II by one ELISA test (Abbott 3.0, recombinant HIV-I/HIV-II antigens, Abbott Diagnostics Division, detecting IgG and IgM simultaneously) and positive results were confirmed using a second ELISA (Enzygnost, synthetic *env* peptides, Behring) as well as a rapid assay, either the rapid agglutination test (Capillus latex agglutination assay, using recombinant polypeptides from the *env* region, Cambridge Biotech Limited); or the rapid ELISA (Clonatec Rapid HIVI,II Ab using two synthetic peptides from the, gp41 for HIV-I and gp36 for HIV-II, regions). A commercial western blot assay (New LAV-blot I, Diagnostics Pasteur) was performed on serum samples that showed discrepant serology results (i.e. if one or both the confirmatory assays were negative). The western blot was considered positive if the specimen reacted with at least two of the following bands: gp160, gp120, gp41, p24. Any other pattern of bands was interpreted as representing an indeterminate result.

4.2.3 DNA preparation

Peripheral venous blood from each seropositive patient was collected in sterile EDTA, and stored at 4°C for not longer than 48hr before processing. Negative control blood samples were included among the patients' blood samples so that no bias was introduced. The PBMCs were isolated and DNA prepared using the lysis method as described by Kawasaki (1990) with a few alterations.

One hundred microlitres of whole blood was mixed with 1,4ml of TE buffer (10mM Tris-HCl, 1mM EDTA, pH7.6) to lyse the red blood cells, and centrifuged for 45sec at 13,000g in a Beckman E microfuge. The pellet of concentrated PBMCs was resuspended in 1ml TE buffer by vortexing,

and washed by repeat centrifugation. This “washing” procedure was repeated until the supernatant fluid was clear. Normal blood contains in the order of 5000 white cells per microlitre, thus the pellet from 100µl of whole blood will contain 500 000 or more nucleated cells. The final pellet was resuspended in 300µl of K-buffer (50mM KCl, 10mM Tris-HCl, 2,5mM MgCl₂ pH8.3, 0,5%(w/v) Tween-20 and 100µg/ml of fresh proteinase K) and incubated for 2hr at 56°C to lyse the PBMCs and release DNA. The proteinase K was inactivated at 95°C for 10min.

The DNA preparations were stored at 4°C and an aliquot of the whole blood was kept at -20°C. To assess the quality of the DNA preparation and to verify that the DNA was suitable for PCR analysis, PCR was performed using primers, SGDQ1 and SGDQ2, to amplify the human leukocyte antigen DQα (HLA-DQ-α) (Innis and Gelfand, 1990). Only samples that were positive for this single copy cellular gene were used in the HIV PCR assay.

4.2.4 HIV PCR test procedure

PCR test procedures were performed using a University PCR ESU temperature cycler. Reaction solutions contained 200µM of each of dATP, dCTP, dGTP (Boehringer Mannheim) and the optimum concentrations of dUTP as established in chapter 3, 50pmol of each different set of HIV-I,II primers, and 1X reaction buffer (Boehringer Mannheim). Ten microlitres of the cell lysate was then added to make a final reaction volume of 50µl. The magnesium and *Taq* DNA polymerase concentrations were adjusted to the optimal concentration for each primer set, as previously determined in chapter 3. Each patient's DNA sample was run in duplicate together with a negative DNA control, a positive DNA control and a negative reagent control. Two HIV-I and one HIV-I,II specific primer pairs, SK38,39; SK29,30 and SK145,431 (as described in

chapter 3, table 5) were used for DNA amplification. Primer locations on the HIV genome are shown in figure 20.

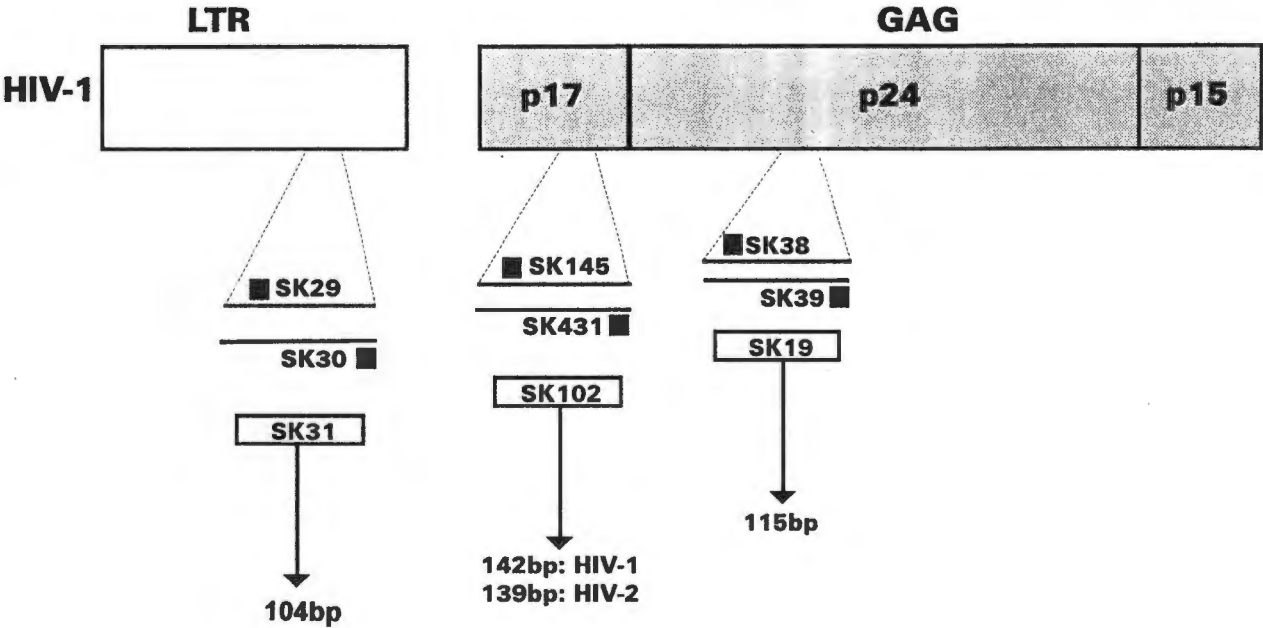


Fig. 20 Location of primers and their probes, used in this study, on the HIV genome

The following cycling parameters were employed for the DNA amplification: DNA was denatured by heating to 94°C for 2min. The primers were then allowed to anneal to their target sequences at 50°C for 30sec, and extension of the target sequence was achieved by increasing the temperature to 72°C for 60sec. The DNA duplexes were denatured again by heating the reaction to 94°C for 20sec. The cycle, commencing at this denaturation step, was then repeated 40 times.

All pipetting steps prior to amplification were performed using plugged tips. The PCR reagents and DNA isolations were prepared in separate rooms (from where PCR products were generated and analyzed) to decrease the likelihood of false positive reactions caused by “carry-over” or specimen cross contamination.

The PCR products were analyzed by agarose gel electrophoresis and Southern blot hybridization, as previously described in chapter 3.2.4. The PCR was only considered positive if the DNA sample was reactive with at least two of the HIV primer pairs, in duplicate, after hybridization with the respective DIG-labelled probes (Fig. 21).

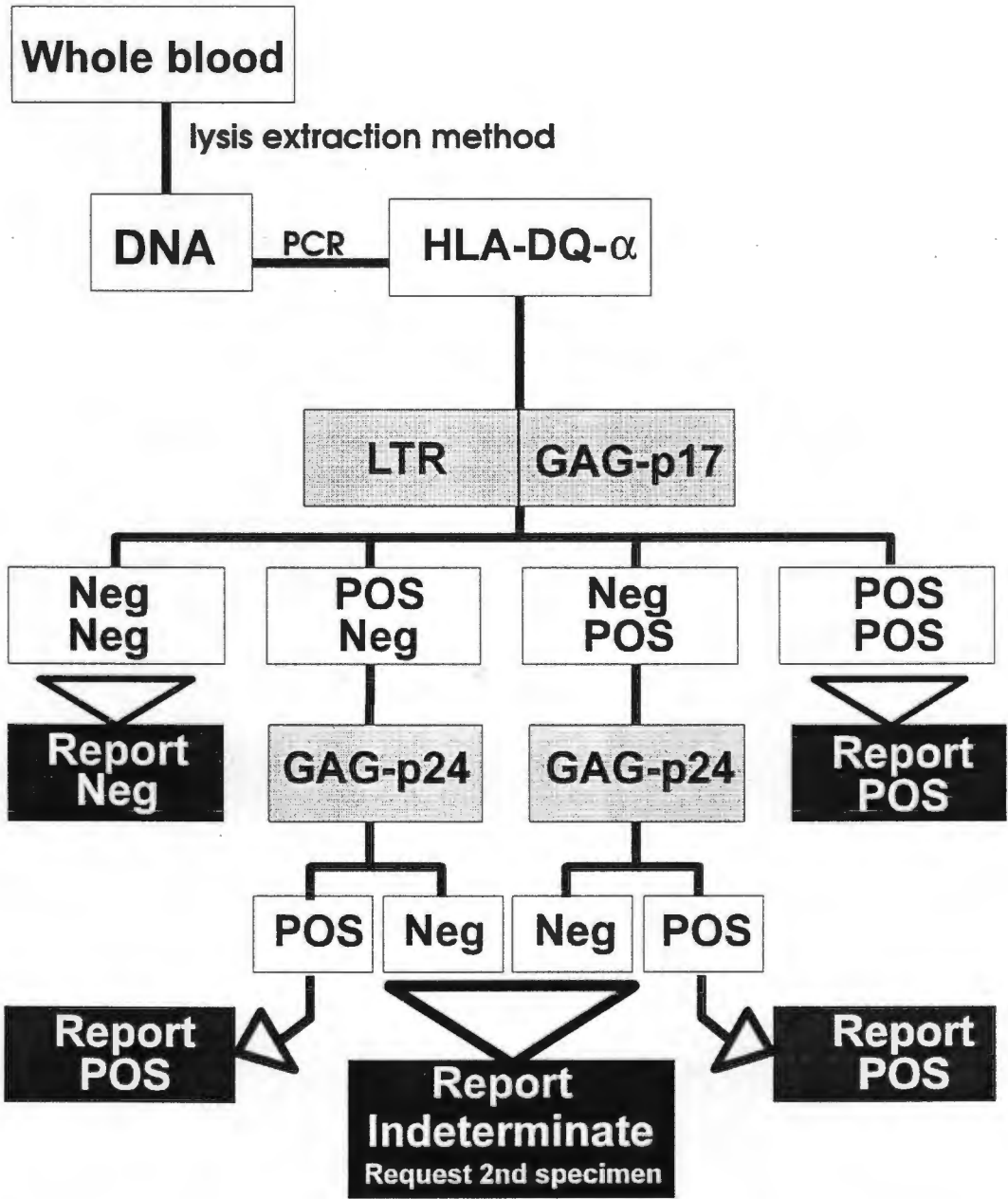


Fig. 21 Strategy for detection of HIV by PCR . Reactivity with two different primer sets are required, before a positive result is reported.

4.3 Results

4.3.1 Serology results

Of the 153 patients screened, 138 were found to be HIV seropositive by the two different ELISAs (Abbott 3.0 [Fig. 22] and Enzygnost Behring) as well as the rapid screening test.

A

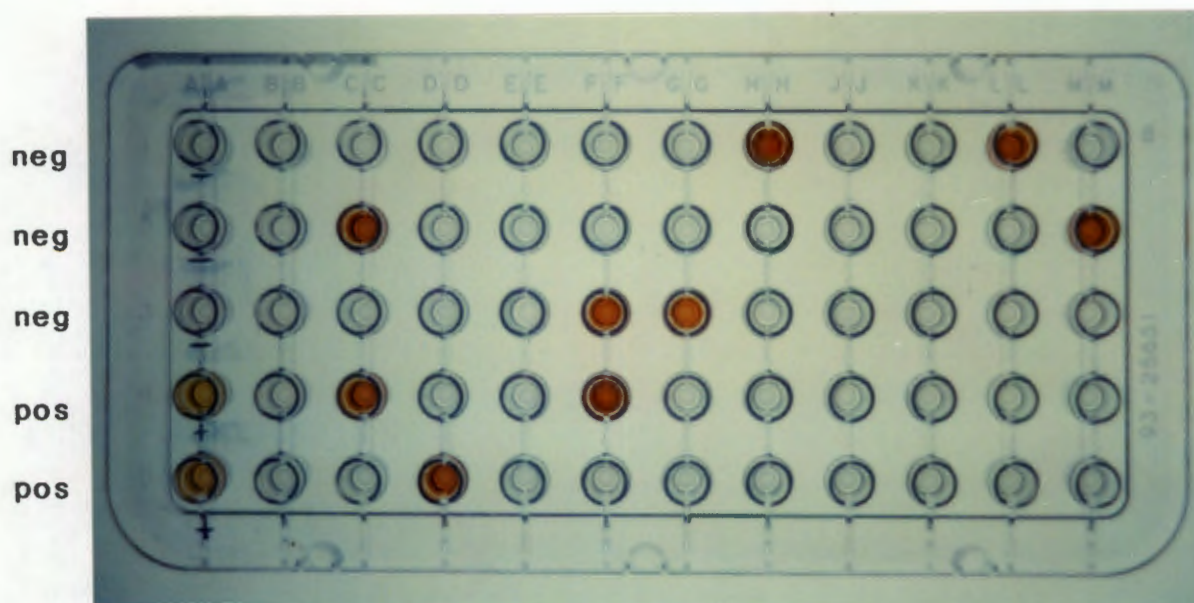


Fig. 22 ELISA (Abbott 3.0) antibody screening test for HIV specific antibodies. Row A on the plate contains 3 negative and 2 positive controls, and the rest of the wells are patient sera. The orange coloured wells are an indication of sera containing HIV specific antibodies.

Eleven patients were HIV seronegative by both the Abbott 3.0 ELISA and the rapid agglutination test; 2 gave indeterminate serology results, and two were not tested for HIV antibodies because of insufficient volume of blood. The two sera with indeterminate or discrepant serology results both gave low positive results on the ELISA screens and the rapid

agglutination test was negative. Western blot analysis was performed on these two sera, and both were found to be negative (the specimens did not react with any of the HIV-specific proteins on the western blot strip).

4.3.2. Amplification results

4.3.2.1 HLA-DQ- α PCR results

It was demonstrated that all the samples tested contained amplifiable DNA since the HLA-DQ- α gene could be amplified. (The absence of an HLA product, after EtBr staining would indicate either the presence of an inhibitor of the PCR or an insufficient amount of cellular DNA.) The HLA primers yielded a 254bp fragment (Fig. 23).

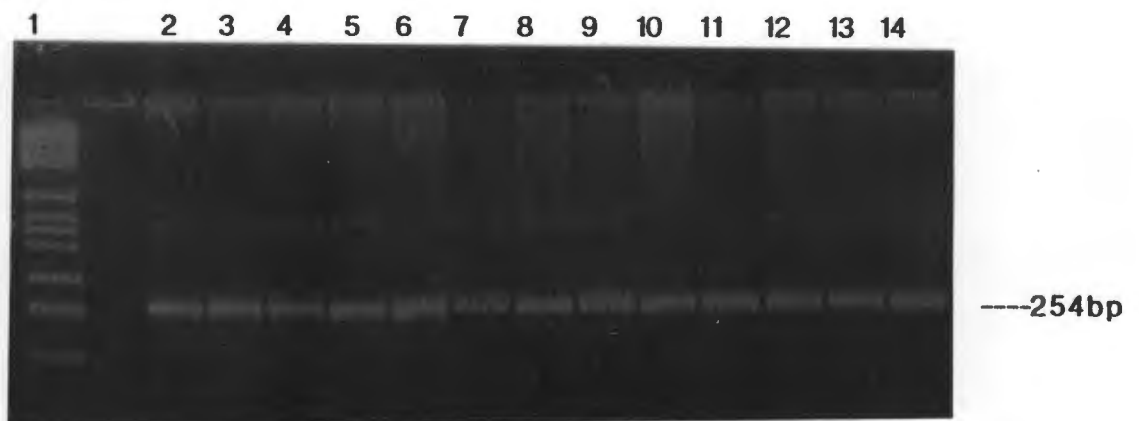


Fig. 23 Amplification results for the HLA-DQ- α gene in PBMCs from patient samples.

Lane 1 MWVI; Lanes 2-14 cell lysate from 13 patients.

4.3.2.2 HIV proviral DNA results

The criteria for a positive HIV PCR result were that all samples were required to be reactive, in duplicate, with at least two different HIV primer pairs (Fig. 24).

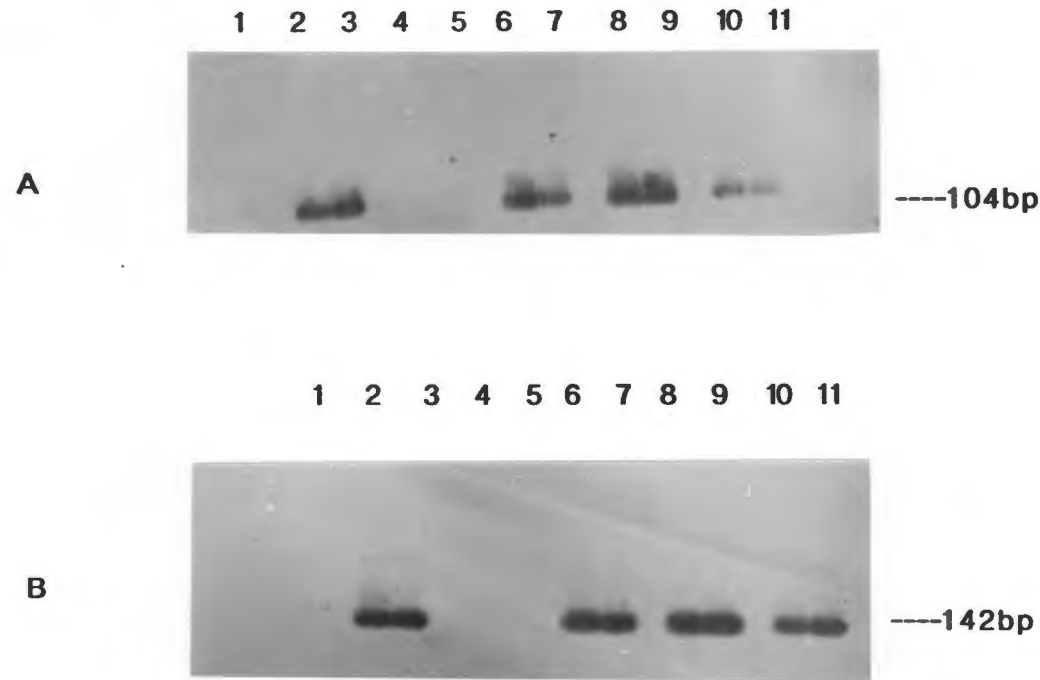
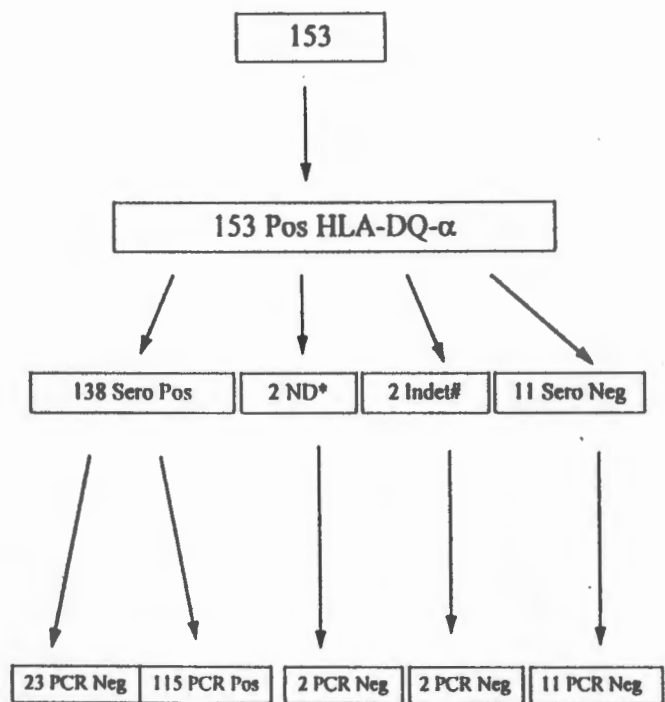


Fig. 24 Detection of HIV-1 DNA in PBMC by using PCR and oligonucleotide hybridization. Cell lysates were amplified with primers **SK29,30 (A)**, and **SK145,431(B)**. Lanes 1 Blank control; Lanes 2-3 pos patient controls; Lanes 4-5 Neg DNA controls; Lanes 6-7 patient no1; Lanes 8-9 patient no2; Lanes 10-11 patient no3.

All 11 seronegative specimens were negative by PCR with all three HIV primer pairs. The two serology-indeterminate and western blot negative specimens were also PCR negative with all three HIV primer pairs. The two specimens that were insufficient for HIV serology, were also PCR negative with all three primer pairs. Of the 138 seropositive specimens, 115 were PCR positive with at least two HIV primer pairs, and the remaining 23 were PCR negative (Fig. 25).



* Not Done # Indeterminate Serology Result

Fig. 25 PCR test results of 153 patients compared with their HIV serology status.
 * not done # indeterminate serology result

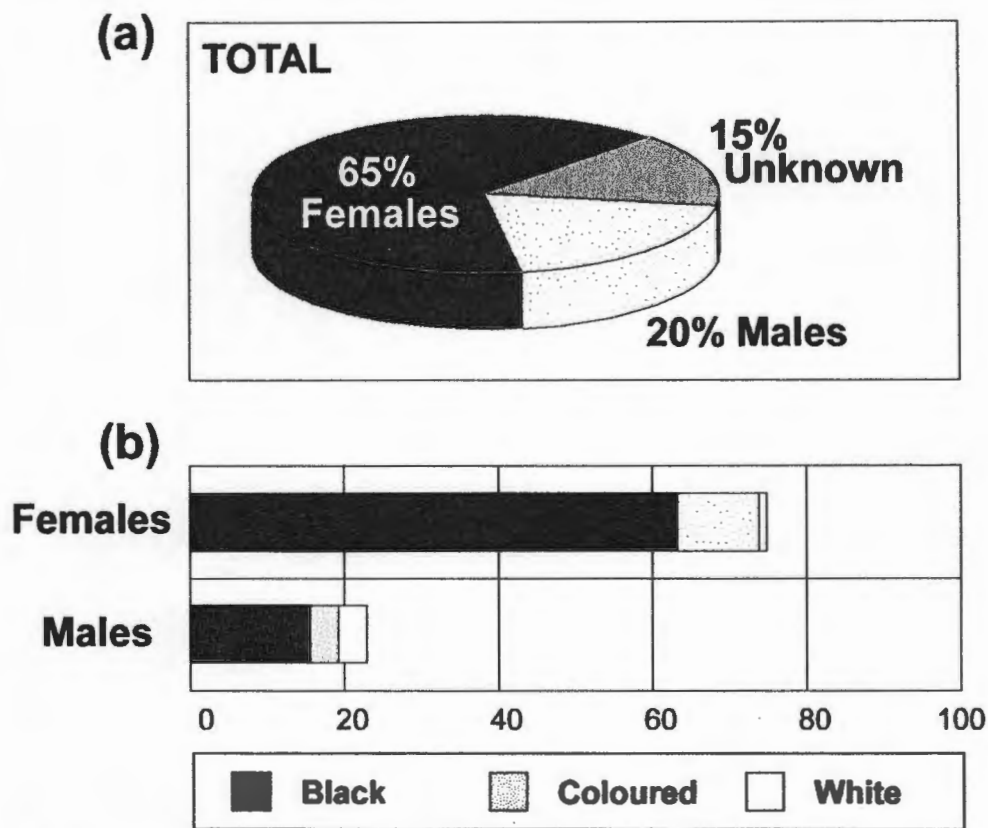


Fig. 26 Analysis of the 115 PCR positive specimens identified in this study, with (a) presenting the total amount of PCR positive specimens according to the sex of the patients, and (b) representing the male and female population of the positive specimens.

Figure 26 shows the female to male ratio of those samples that were PCR positive, indicating 65% of these patients to be female and only 20% male. Sixty-four percent of the PCR positive males were black, 18% coloured and 18% white. In contrast, 82% of the PCR positive female

Different combinations of the three HIV primer pairs were used to determine the PCR status of these 138 samples. Initially 25 samples were tested with the nested *env* (C₁C₂, SK 68,69) and *gag*-p24 (SK38,39) primer pairs and a good correlation was obtained as all 25 samples were positive with both primer pairs (Fig. 27A). However, further testing with the same primer combination indicated that contamination had been introduced (shown by a positive reaction in the blank reagent control and in the negative DNA control). As a result, the *env* primer pair was discarded and eliminated from further tests. In future tests the *env* primer set was replaced with the LTR (SK 29,30) primer set. Twenty-seven sera were tested with this combination (Fig. 27B) and identical results (26 positive, 1 negative) were obtained with both primer pairs. Because there is a possibility that *gag*-p24 will not detect HIV-II sequences, it was decided to include a *gag*-p17 (SK145,431) primer pair in the assay. Four sera assayed with each of the two different *gag* primer pairs (Fig. 27C) did not reveal any difference. The *gag*-p17 (SK 145,431) primer pair was combined with the LTR (SK 29,30) primer pair to assess the final 82 specimens and identical results (Fig. 27D) were obtained (61 positive and 21 negative). This combination of primers were therefore considered to be well suited for diagnostic use.

A

		GAG-p24 SK38,39	
ENV SK68,69		POS	NEG
	POS	25	0
	NEG	0	0

B

		GAG-p24 SK38,39	
LTR SK29,30		POS	NEG
	POS	26	0
	NEG	0	1

C

		GAG-p24 SK38,39	
GAG-p17 SK145,431		POS	NEG
	POS	3	0
	NEG	0	1

D

		GAG-p17 SK145,431	
LTR SK29,30		POS	NEG
	POS	61	0
	NEG	0	21

Fig. 27 PCR results of the different HIV primer combinations used to detect the provirus in the PBMCs of patients tested in this study.

4.4 Discussion

After optimization of the HIV PCR assay (chapter 3) for our laboratory, a number of routine specimens were analyzed in this system to establish the degree of correlation between PCR results and serology results. When PCR is developed for a diagnostic assay, it is essential to use several primer pairs in order to identify the best single primer pair for that specific target organism. However, the heterogeneity of HIV-I also stresses the need for different primer pairs, to ensure the detection of all HIV variants. All primer pair combinations in this study showed a good correlation. The SK38,39 (*gag*-p24) and SK68,69 (*env*) primers were initially used for this study as they were well described in the literature (Ou *et al.*, 1988; Rogers *et al.*, 1991). The *env* primer pair was later omitted from this study as a result of contamination, and was replaced by the LTR primer set SK29,30 and the *gag*-p17 primer set SK145,431 (Borkowsky *et al.*, 1992; Kellogg and Kwok, 1990). Kwok and Sninsky (1993) mention that they and others have analyzed more than 1000 samples using the primer combination of SK38,39 and SK462,431 (both in the *gag*-region) and found greater than 98% concordance between the two pairs. SK462 is a derivative of the previously described SK145, which was initially designed to amplify both HIV-I and HIV-II, but as more isolates were sequenced it became apparent that SK145,431 will only amplify a subset of HIV-IIs. The modified primer pair that replaces SK145 with SK462 will now amplify HIV_{mal}-like sequences (HIV-I with two mismatches).

It was found that 83% of the seropositive patients were also PCR positive, and 17% were PCR negative. The reasons for the 17% PCR negativity was not as a result of insufficient DNA because amplification for the HLA-DQ- α gene was positive. Analysis of the patient data revealed that all 23

PCR negative (but seropositive) samples were from infants less than 15 months old. The seropositive status of these PCR negative infants could be a reflection of the mother's antibody status since maternal antibodies can persist in the infant for 15 to 18 months. As these infants were not part of a prospective study, it was not possible to obtain follow-up blood samples to determine their true antibody status after 18 months of age. Not all babies born to HIV-positive mothers are infected. The transmission rate of HIV-I from mother-to-infant is estimated at 20-65% (Soeiro *et al.*, 1992). We did in fact find that 27 seropositive infants were also PCR positive. A fuller investigation of the application of PCR to the diagnosis of infants is given in chapter 5.

All the seronegative samples were also PCR negative. These samples were taken from: 1) adult patients with no apparent risk for HIV infection, but for whom pathologists had requested a screening test to exclude an HIV infection, 2) infants who were born to HIV positive mothers, but who had remained seronegative after 12 months of age, and 3) husbands whose wives were HIV positive, but who themselves had remained seronegative. Thus, the correlation between negative PCR results and negative serology was not unexpected. Cases have been described where the serology is negative but the PCR is positive. For example, in blood samples from patients undergoing seroconversion, PCR can detect HIV proviral DNA in the lymphocytes before an ELISA can detect any HIV specific antibodies (Bruisten *et al.*, 1992; Imagawa *et al.*, 1989). We did not identify any such cases in this study as all PCR positive samples were also HIV-antibody positive in the initial screening. However, a few seroconversion cases have since been identified in our laboratory (unpublished data). Positive HIV diagnosis was based on clinical grounds and the initial detection of only the p24 HIV specific band on the western blot assay, followed by the appearance of more HIV specific bands in subsequent specimens from the same patient. Antibodies to the

gag proteins (p18, p24, and precursor p55) are generally the first to appear in a patient with recent HIV infection. These antibodies are also the first to disappear in advanced cases of AIDS. An early seroconversion is generally reflected on the western blot assay as a pattern with antibodies to the p24 and/or gp160. Antibodies to the other virus-specific proteins will become detectable as the disease progresses. The western blot of indeterminate sera was negative for all HIV-specific bands, which could indicate that the initial serology was probably not HIV specific.

Indeterminate PCR results (e.g. where only one of the duplicate PCR reactions are positive after probing) did not pose a problem in this study, but such results may occur, for example in samples containing very low copy numbers of HIV. The safest way to deal with samples of this nature, is to request a follow-up sample (within a week or two), and to repeat the PCR. Simmonds *et al.* (1990) showed that the frequency of infected PBMCs from a patient with AIDS was about 1 per 500, and from an asymptomatic patient it was about 1 per 10 000. In our case (using 500 000 cells from 100 μ l) there were between 50 to 1000 infected cells per 100 μ l of whole blood.

The majority of PCR positive specimens were obtained from female patients. The gender bias is probably a reflection of the sources of clinical material used in this study. These comprised hospitals with ante-natal clinics and facilities for paediatric care. Often clinically ill infants are first shown to be infected with HIV and this leads to subsequent identification of HIV positive mothers, thus more females than males were screened. Worldwide, heterosexual spread is the most common way in which HIV is transmitted, followed by vertical spread from infected mother to infant. The large numbers of infected women and babies identified in this study is an indication that heterosexual transmission is also prevalent

in the local community. The majority of all the PCR positive patients were black. Again this might be related to the fact that most of the specimens were received from state hospitals where patient care is provided free, or at minimal cost, for pregnant women and their children. These hospitals serve mainly indigent members of the black and coloured communities. The few specimens from white patients were received from private pathologists in the Cape Town area who referred their positive sera to our laboratory in order to verify the results that they had obtained.

A few important technical notes were made during this study that help to achieve maximum sensitivity when HIV PCR is performed, and include the following: 1) Whole blood specimens should be processed as soon as possible after collection of the specimen, as long periods of storage of the whole blood at room temperature or 4°C can affect the yield of amplifiable DNA. It was our experience, however, that whole blood could be stored at -20°C for 12-24 months and still produce amplifiable DNA. 2) Adequate washing procedures during the extraction of the PBMCs from whole blood is essential, as any trace of haeme-related compounds may inhibit amplification. 3) The inclusion of two different HIV primer pairs is advised as this increases the sensitivity and ensures greater likelihood of detecting any HIV-1 variants. The inclusion of sufficient positive and negative controls and confirmation by hybridization are essential at this stage, since PCR procedures are prone to contamination and amplification of non-specific DNA sequences.

The PCR technique thus provides a highly sensitive and specific alternative to HIV culture and p24 antigen detection for the direct detection of HIV, being particularly useful in resolving the infection status of indeterminate serology individuals and babies born to HIV-infected mothers (chapter 5). It can also serve as a handy molecular tool to characterize genetic variability (chapter 6).

Chapter 5

Application of HIV PCR in amplification of sequences from infants born to seropositive mothers

5.1 Introduction

Infection of children with human immunodeficiency virus type I has been recognized since 1983 (Rubinstein *et al.*, 1983), and the number of HIV-I infected children has increased in recent years in parallel with the increase in HIV-I infected women of childbearing age. The overwhelming majority of childhood HIV-I infections are believed to be transmitted pre- or perinatally from infected women (Fallon *et al.*, 1989). Neonates born to HIV seropositive mothers may demonstrate signs associated with HIV infection or AIDS within the first year of life. These clinical signs can include: 1) pneumonitis 2) oral candidiasis 3) diarrhoea 4) gastrointestinal complaints 5) swollen lymph nodes 6) non-pruritic macular erythematous rash 7) acute central nervous system disorders (encephalitis) 8) headache, muscle aches, sore throat, low- or high-grade fever (Levy, 1984). These symptoms could persist for 3 weeks, with lymphadenopathy and failure to thrive for many months. By the time these infected infants reach the age of two, about three-quarters of them will present with clinical symptoms of the disease (Gibb and Wara, 1994). The frequency of materno-fetal transmission of HIV-I varies from 20% to 65%, with African countries having the highest transmission rates (Ryder *et al.*, 1989; Hira *et al.*, 1989; Soeiro *et al.*, 1992; Newell *et al.*, 1992,

also see chapter 1.7.1.5 for further information), but accurate diagnosis of infection in these babies is not a simple procedure.

The use of serological methods is inadequate to differentiate infected infants from uninfected infants born to HIV-seropositive women, since the mother's antibodies (IgG) cross into the infant's bloodstream via the placenta, and may persist in the infant for 15 to 18 months. The currently available ELISAs (see chapter 2) cannot distinguish between the passively acquired maternal antibodies (IgG) and antibodies produced by the infant in response to an HIV infection. The detection of other classes of immunoglobulin (IgM and IgA) that do not cross the placenta have been considered for the diagnosis of true infection. IgM antibodies to HIV are produced early in the course of infection but they are only detectable in the blood for a relatively short period of time. IgM may disappear before maternal IgG leaving a time period where it is impossible to distinguish between IgG from infant or mother. The production of IgA occurs shortly after IgM, and remains stable for many years. As IgA antibodies are mainly secreted by cells of the mucous membranes of the body, they can be detected in mucous secretions as well as in the blood. However, it has been shown that HIV infection is not reliably detected by the IgA antibody assay during the first three months of life (Gaetano *et al.*, 1987 and Martin *et al.*, 1991). Thus, assays that directly detect the presence of infectious virus, or viral components, are required for the diagnosis of HIV infection in infants.

The detection of viral proteins such as the p24 core antigen, can also be considered as evidence of viral infection. However, p24 antigenaemia is not always observed in all infected infants, and the assay lacks sensitivity (Borkowsky *et al.*, 1992). Viral culture is a specific and sensitive means of diagnosis but the detection of HIV by this method can take from 1 to 4

weeks or longer to complete, is expensive and requires special biosafety precautions (Petru *et al.*, 1992).

Ideally, a laboratory test should permit diagnosis as soon as possible after infection, and thus allow antiviral or other prophylactic therapy to be administered at the earliest opportunity. In the majority of cases babies are infected at the time of confinement and it is not known precisely how soon thereafter the infection can be diagnosed with accuracy. The sensitivity of viral tests for HIV is often less than 50% in the first weeks of life, and even tests like HIV PCR and culture are highly sensitive only after 2 months of age (Krivine *et al.*, 1990).

This chapter describes the use of PCR to detect the presence of HIV proviral DNA in PBMCs from infants less than 15 months of age, and examines the possible advantages of PCR as opposed to serological assays for the diagnosis of HIV infection.

5.2 Materials and Methods

Forty-five infants, all less than 15 months of age and born to HIV-seropositive mothers were included in this study. Their sero-status was determined as described in chapter 4.2.2 by using the protocol by which all initial screening tests were done with one ELISA (Abbott 3.0 recombinant HIVI,II) and positive results were confirmed using a second ELISA (Enzygnost Synthetic *env* peptides, Behring) and a rapid HIV assay (Capillus Latex Agglutination using recombinant polypeptides from the *env* region, Cambridge Biotech Limited).

Peripheral blood mononuclear cells from these infants and from healthy adult donors (as controls) were concentrated from whole blood, and DNA

was extracted using the method described in chapter 4.2.3. All DNA preparations were subjected to PCR with the HLA-DQ- α primer pair to verify that the DNA was suitable for further PCR analysis.

Two sets of primers and their probes were used for HIV-I, and/or HIV-II amplification: SK29,30 (Ou *et al.*, 1988) and SK145,431 (Kellogg and Kwok, 1990). A third HIV-I primer pair, SK38,39 (Ou *et al.*, 1988) was available in case any discrepant PCR results were obtained (chapter 3.2.1). In order to be considered positive, the samples were required to yield positive results with at least 2 different HIV primer pairs, in duplicate, after hybridization with the respective DIG-labelled probes.

Ten microlitres of cell lysate was amplified for 41 cycles in a 50 μ l reaction mixture. The PCR test procedure and cycling parameters were performed according to the optimized conditions as previously described in chapter 4.2.4. All the samples were tested in duplicate and each PCR run included a positive DNA control, a negative DNA control and a negative reagent control. Analysis of the PCR products included agarose gel electrophoresis and Southern blot hybridization with DIG-labelled oligonucleotide probes (see chapter 3.2.4).

5.3 Results

5.3.1 Comparison of HIV-PCR results with serology

Serological assays indicated that 42 of the 45 infants had antibodies to HIV-1, and three of the infants were seronegative. Analysis of the DNA isolated from the PBMCs from all 45 infants gave positive amplification of the HLA-DQ- α gene indicating that there was sufficient DNA to amplify and there were no inhibitors to the amplification.

The 3 seronegative samples also tested negative for HIV proviral DNA by PCR with 2 different HIV primer pairs and their probes. Twenty seven (64%) of the 42 seropositive infants were also PCR positive, in duplicate, after hybridization with two different HIV probes, as shown in figure 28. The remaining 15 (36%) seropositive infants were negative for HIV proviral DNA by PCR.

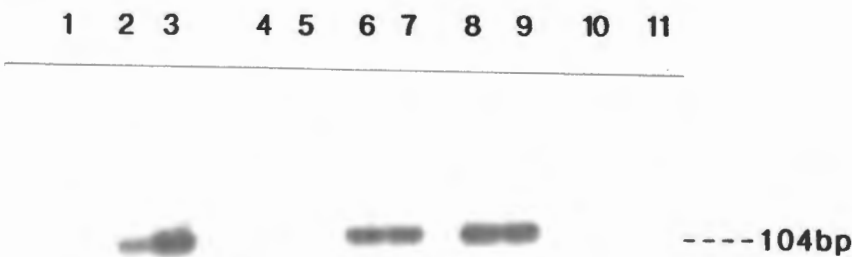


Fig. 28. Detection of HIV-1 proviral DNA in PBMCs of infants (< 15 months of age) by using PCR and oligonucleotide hybridization. Cell lysates were amplified with primers SK29,30. Lane 1, blank control; lanes 2-3, pos patient control; lanes 4-5, neg DNA control; lanes 6-7, patient no1; lanes 8-9, patient no2; and lanes 10-11, patient no3.

Forty-three of the 45 infants were evaluated using the primer pair combination of SK29,30 and SK145,431 (Fig. 29A), and the remaining two were evaluated with the primer combination of SK29,30 and SK38,39 (Fig. 29B). Both of these primer combinations gave perfect correlation, as no discrepant results were obtained.

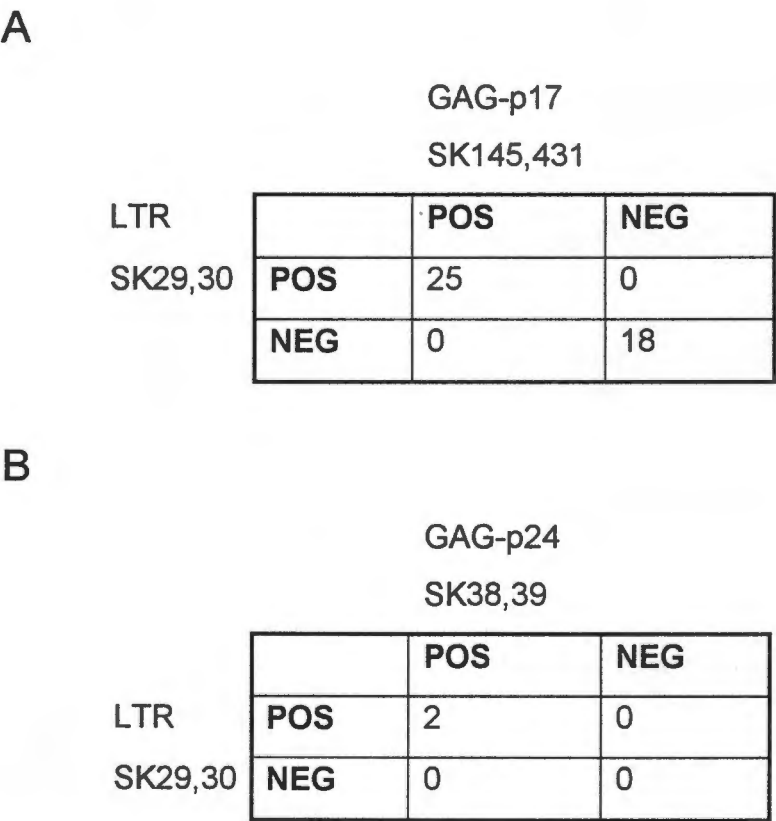


Fig. 29 Results of HIV PCR with the different primer combinations: A) SK29,30 and SK145,431, B) SK38,39 and SK29,30.

Figure 30 (next page) illustrates the comparison of the serostatus with the PCR-status of all 45 infants.

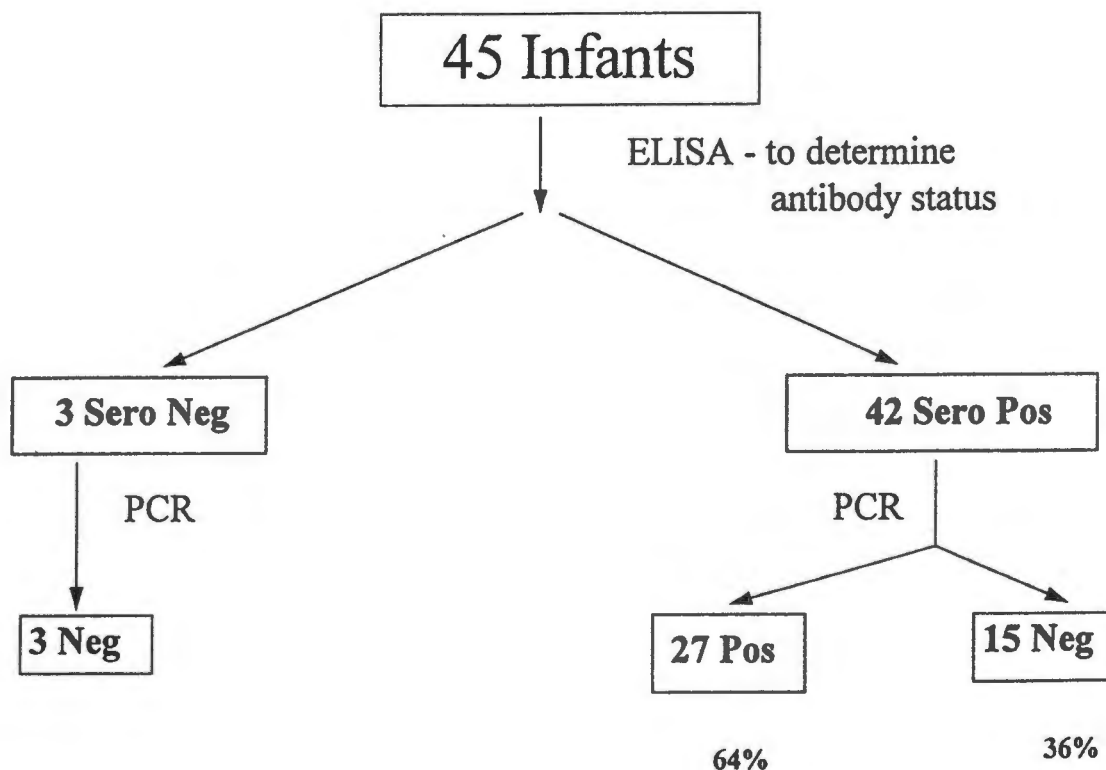


Fig. 30. Results of assays for HIV serum antibodies and proviral DNA performed on 45 infants less than 15 months of age, born to HIV sero-positive mothers.

5.3.2 Collation of patient data with HIV test results

The 3 seronegative, PCR-negative samples were repeat specimens from infants 13, 12, and 14 months old respectively. All three had been tested previously and found to be HIV seropositive; two at the age of 3 months and one at birth. No clinical data was available for two of these infants, but the third one was reported to be clinically well at the time of follow-up.

For those infants who were seropositive, the PCR status was examined with respect to the age of the infants and the clinical data that was available at the time that the specimens were received (Table 10).

Table 10 Combined data of the 42 seropositive infants.

Age group	Baby no	Age	Gender	Sero-status	PCR-status	Clinical data
0-3 months	1	1 day	F	●	□	⊕
	2	1 day	M	●	□	⊕
	3	5 days	F	●	□	⊕
	4	7 days	M	●	□	adoption program
	5	1 month	M	●	●	⊕
	6	1 month	M	●	●	⊕
	7	1 month	F	●	●	⊕ ⊛ tuberculosis
	8	2 months	M	●	●	⊕ ⊛
	9	2 months	M	●	□	⊕
	10	2,5 months	M	●	●	gastroenteritis
	11	3 months	M	●	●	<i>Pneumocystis pneumonia</i>
	12	3 months	F	●	●	previous HIV-seropos
	13	3 months	M	●	□	⊕
	14	3 months	M	●	□	⊕
	15	3 months	M	●	□	⊕
4-6 months	16	4 months	M	●	●	⊕ hepatosplenomegaly lymphadenopathy, gastro
	17	4 months	M	●	●	pneumonia, diarrhoea
	18	4 months	M	●	●	⊕ ⊛ full blown AIDS
	19	4 months	M	●	●	⊕
	20	4 months	M	●	□	⊕
	21	4 months	F	●	□	⊕
	22	4 months	F	●	□	⊕
	23	6 months	M	●	●	lymphadenopathy hepatosplenomegaly
	24	6 months	M	●	●	prev HIV seropos
	25	6 months	F	●	●	pneumonia, candida
	26	6 months	F	●	●	diarrhoea, hepatomegaly
	27	6 months	M	●	□	⊕

Age group	Baby no	Age	Gender	Sero-status	PCR-status	Clinical data
7-9 months	28	7 months	F	●	□	⊕
	29	7 months	M	●	●	⊕
	30	8 months	F	●	□	no data available
	31	8 months	M	●	●	lymphadenopathy hepatosplenomegaly
	32	9 months	M	●	●	cryptosporidium diarrhoea
	33	9 months	F	●	●	prev HIV seropos
	34	9 months	M	●	□	⊕
10-12 months	35	10 months	M	●	●	⊕ prev HIV seropos
	36	12 months	F	●	●	⊕ prev HIV seropos
	37	12 months	M	●	●	prev HIV seropos
	38	12 months	F	●	●	lymphadenopathy
	39	12 months	M	●	●	prev HIV seropos
	40	12 months	F	●	●	failure to thrive, enlarged lymph nodes
>13 months	41	13 months	F	●	●	hepatosplenomegaly pneumonia gastroenteritis
	42	14 months	F	●	●	tuberculosis, pneumonia

⊕=mother HIV positive, ⊗=father HIV positive,

M=male, F=female, ●=positive, □=negative

The 15 seropositive but PCR-negative infants included 8 who were 3 months of age or younger, 4 who were >3 months but <6 months old, and 3 who were >6 but <9 months old. Nine of these infants were males and the remaining 6 were females. The only available clinical information regarding all the infants was that their mothers were HIV seropositive. The mother of one of these infants was only diagnosed as being HIV seropositive after her baby had been tested seropositive. This baby was part of an adoption program for which HIV screening was required.

The group of 27 seropositive and PCR-positive infants consisted of 17 males and 10 females which was not significantly different from the

gender ratio seen in uninfected (PCR-negative) babies. This group included 7 infants who were between 1 and 3 months old, 8 who were >3 but <6 months, 4 who were >6 but <9 months, 6 who were >9 but <12, and 2 who were between 12 to 15 months old (Fig. 31). Clinical data was available for only 15 of these infants. Signs indicative of immunosuppression and opportunistic infections were associated with a number of HIV-infected babies of 3 months and older, and included the following: *Pneumocystis carinii* pneumonia, tuberculosis (*Mycobacterium tuberculosis*), cryptosporidiosis, chronic diarrhea, candidiasis, lymphadenopathy, full blown AIDS, hepatomegaly, failure to thrive, hepatosplenomegaly, and enlarged lymph nodes. Positive PCR results were obtained for 11 infants who were screened for HIV because their mothers and/or fathers had been diagnosed as being HIV seropositive. Four of the seropositive babies were under a week old and they were also PCR negative. However, positive PCR results were obtained for 3 babies of only 1 month of age. All 8 infants older than 9 months were PCR positive as well as seropositive.

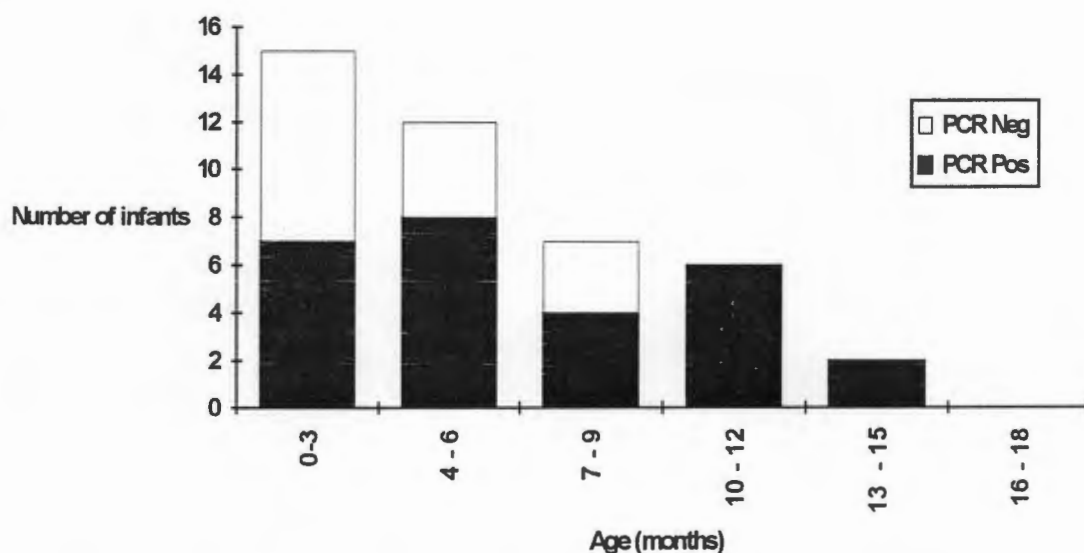


Fig. 31 Illustration depicting the number of PCR-positive assays (dark blocks) in seropositive infants of various age categories (given in months).

5.4 Discussion

The primers that were evaluated and chosen in chapter 3 and 4 (SK29,30; SK145,431; SK38,39) gave reliable and reproducible results when applied to diagnosis of HIV in infants. One should be aware of problems that might arise because of incompatibility between the primers and new divergent genetic strains. However, a recent study performed by Van Rensburg and Engelbrecht in South Africa, indicated that different gag primers (SK38,39; gag22,Sk39; gag a/b,gag c/d nested), LTR primers (SK29,30), and *env* primers (SK68,69) were able to detect all HIV-I subtypes (B, C, and D) presently known to occur in the Western Cape (unpublished data, Vth International Congress on the Impact of Viral Diseases in the Developing World, July 1995).

In cases of neonatal infection with HIV, an early diagnosis is desirable for the clinical management of the infected infant especially when antiviral treatment is an option, as this treatment may be more effective if it is given as soon as possible after infection. PCR methodology is increasingly being used to determine the presence or absence of HIV infection in neonates and is preferred to viral culture because results may be obtained far more rapidly, and the technique requires a smaller sample size.

Because of the presence of maternal antibodies, serology results are inadequate to determine true infection in babies born to seropositive mothers. In this study we were able to confirm infection by the detection of proviral DNA in 64% of the seropositive infants examined. Fifteen of the PCR positive infants (but none of the PCR negative infants) displayed clinical symptoms indicative of an HIV infection, and these babies were all older than 3 months. The majority of perinatally infected children develop

HIV associated symptoms within the 1st year of life (Scott *et al.*, 1990). Early manifestations in most of these infants include oral candidiasis, splenomegaly, hepatomegaly, lymphadenopathy, diarrhoea and failure to thrive. However, some infants will present with more severe AIDS-defining illnesses during this time. Friedland and McIntyre reported in 1992 when they studied perinatally acquired HIV infection in infants born to HIV positive mothers at the Baragwanath Hospital, that most of these infants became symptomatic before the age of 6 months and over 70% of them presented with the clinical features described above. In the case of adults, clinical signs of HIV infection only become evident after the "latent" stage of the disease, that can last from 8 to 15 years, when the virus load in the body increases and more cells become infected (Schoub, 1994).

A PCR negative status in the presence of positive serology does not necessarily exclude infection with HIV, especially in very young babies. It is recognized that in babies under 2 months of age the sensitivity of the PCR test is less than 50% (Krivine *et al.*, 1990). This may reflect very low levels of virus in the peripheral blood or that the virus is confined in lymphoid organs. If there is an initial lag period before the virus begins to replicate, the number of infected lymphocytes (or PBMCs) may be very low, thus reducing the probability of their presence in the small samples of blood that can be taken from very young babies. These considerations are relevant in the assessment of the PCR negative status of the four babies who were under one week of age. It has been reported that an increase in viral load in the PBMCs seems to occur during the first few weeks of life, as indicated by an increase in the p24 antigen levels (Krivine *et al.*, 1990 and Borkowsky *et al.*, 1992). Our study group did not include any infants of between 1 and 4 weeks of age, but indeed 5 of the 6 babies aged between 1 and 2,5 months gave PCR positive test. This suggests that the single PCR negative result in this group is likely to be a

true indication of the absence of infection. It would nevertheless be recommended that such an infant be re-tested at a later age to confirm its true HIV status. The other 10 infants who were seropositive but PCR negative were older than 3 months, and one could therefore probably predict with greater confidence that they were not infected.

The 3 seronegative infants who also tested PCR negative are examples of infants who had lost their passively acquired maternal antibodies, since they had previously been HIV seropositive when tested at birth to 3 months of age. Only by repeat assays at the age of 13 months could the HIV negative serostatus of these infants be verified. The absence of true infection in these infants could probably have been determined at least 10 months earlier with the help of HIV PCR.

In summary, it was shown in this study that PCR can be of good value as a diagnostic assay for the detection of an HIV infection in the majority of infants in the neonatal period although, for reasons given above, it cannot provide proof of infection in newborn babies (<1 week in age). In spite of the high cost of the PCR assay, the overall benefit of an early diagnosis may be sufficient to warrant its being put to more widespread use.

Another possible application for PCR in infants (or adults) is quantitative PCR, for the evaluation of the effectiveness of new drugs and in monitoring disease progression, where an increase in viral load, usually indicates an increase in disease progression (Ho *et al.*, 1989; Alimenti *et al.*, 1994).

Chapter 6

Genetic characterization of the V3-region of HIV-I from a mother and her infant

6.1 Introduction

Nucleotide sequence variation between isolates of HIV-I has been well documented (Burger *et al.*, 1991; Hahn *et al.*, 1984; Ait-Khaled and Emery, 1993; Howell *et al.*, 1991; Milich *et al.*, 1993) and, based on sequence analysis, the virus has been classified into 9 subtypes. Subtypes A to H belong to the so called M group of HIV-I, and the other sequences that do not fit into this group are included in the O group (Myers, 1994). Subtypes A to F exhibit up to 30% variation in their *env* coding sequences. Variability in the *env* gene of HIV-I is especially marked in the region coding for the surface glycoprotein (gp120). Six constant and five hypervariable domains have been identified in this protein. The second conserved domain of 98 amino acids (C2) plays a role in HIV-I infectivity and antibody neutralization (Willey *et al.*, 1986; Ho *et al.*, 1988). The third variable region (also known as the V3 loop) is associated with several functions: infectivity, viral fusion, and syncytium formation (Ivanoff *et al.*, 1992; Freed *et al.*, 1991). The V3 loop is also the principal neutralization domain (PND) that contains T- and B-cell epitopes and is thus the main target of antibody and T-cell mediated immune responses. HIV-I mutants containing only a single amino acid change at residue 128 of the V3 loop, can escape neutralization by antibodies directed against the V3 region. Two observations indicate that V3 has a critical function: 1) the overall length of V3 is conserved,

suggesting that deletions or insertions cannot be tolerated, and 2) random amino acid substitutions in V3 cannot be tolerated (Scott *et al.*, 1990). Genetic analyses of the V3 domain were thus essential in previous strategies aimed at developing an immunogen that would induce neutralizing antibodies to the majority of circulating virus strains (Looney *et al.*, 1988; Scott *et al.*, 1990).

Several studies have shown that there are two different states (phenotypes) of HIV-I in the pathogenesis of AIDS. Slow replicating macrophage-tropic non-syncytium-inducing (NSI) isolates are predominant in the early (primary) asymptomatic stage of HIV-I infection. At later stages, rapid disease progression and rapid decline of CD4⁺ T-cells, as well as reduced survival time after AIDS diagnosis, are usually associated with the presence of syncytium-inducing (SI) HIV-I isolates (Schuitemaker *et al.*, 1992). The acquisition of the SI phenotype is linked to substitutions of acidic or neutral amino acids by more basic amino acids in the V3 loop. Milich *et al.* (1993) examined the pattern of basic amino acid sequences and identified distinct groups of V3 sequences in infected patients that appeared to correspond to the two different stages of infection (the NSI viral phenotype or asymptomatic stage, and the SI phenotype or late stage of infection). The presence of basic amino acids has also been shown to change virus tropism in cell culture (Hwang *et al.*, 1991; Shioda *et al.*, 1992).

The HIV-I virus evolves within an individual during the course of infection (McNearney *et al.*, 1992). Studies have shown that the *env* gene of isolates from unrelated individuals differs by 6% to 22%, and in closely related contacts by 2% to 6% (Burger *et al.*, 1991). Certain host and viral factors may influence this rate of evolution (see chapter 1.6). Following sexual or parental transmission, genetic analysis of HIV-I strains generally shows a genetic distance between the consensus sequences of

viruses from the donor and the recipient, of less than 1% (Wolfs *et al.*, 1992; McNearney *et al.*, 1990). The virus population of the recipients at seroconversion is extremely homogeneous, indicating that a limited number of variants or even a single variant initiates the infection in the new host. In the case of vertical transmission of HIV-I from mother to infant, it has been suggested (Wolinski *et al.*, 1992) that particular variants, such as those that escape the maternal immune response or variants with a tropism for certain cells, can be preferentially transmitted. This suggestion was based on their findings that the HIV variant transmitted to an infant represented a minor genotype in the *env* maternal sequence set. In contrast, however, Swedish researchers (Scarlatti *et al.*, 1993) who compared V3-regions of viruses from infected infants and their mothers, found no evidence of selection of virus variants during transmission. These conflicting data stimulated an examination (in this study) of variants involved in maternal transmission of HIV-I in the local community.

For this purpose, the sequence set of the V3 loop was examined in multiple isolates from one sample of peripheral blood from an HIV-infected mother and compared with that of her 3 month old infant. It was decided to compare sequences from the V3 loop because of the rapid changes that occur in this region of the HIV-I genome compared to other regions. This consequently allows the differentiation between closely related strains. Furthermore, because of the specific functions ascribed to specific sites on the V3 loop, sequence data could possibly yield added information regarding the nature of infectious variants.

6.2 Materials and Methods

Clotted and EDTA blood were obtained from a three month old black male infant and his mother, from Conradie hospital (Cape Town).

The 3 month old infant was diagnosed as HIV-I antibody positive before his mother's HIV status was known. At the time that his first blood specimen was taken, on November the 23rd, the infant had a clinical diagnosis of pneumonia. This blood specimen was positive for HIV-specific antibodies by 2 different ELISA tests and 1 rapid test. A second specimen of clotted blood, as well as an EDTA-blood specimen, was taken on November the 29th and this specimen also yielded a positive HIV- antibody result as well as a positive HIV PCR result (specific LTR and *gag* sequences could be amplified from the extracted DNA). Clinical information given at the time of the second specimen was that the infant had "full blown AIDS". Consequently, a blood specimen from the mother was requested, and this also yielded a positive HIV-I antibody result. No clinical details were given for the mother, and none were available after inquiries at the Conradie Hospital.

6.2.1 Determination of HIV antibody and PCR status

The antibody status of the mother and infant was determined by one ELISA (Abbott 3.0 recombinant HIV-I,II) and confirmed on a second commercial ELISA (Enzygnost, synthetic env peptides, Behring) and on a rapid HIV assay (Capillus Latex agglutination assay, Cambridge Biotech Limited) [chapter 4.2.2].

HIV-I infection in the infant was confirmed by PCR as described in chapter 4.2.3. All DNA preparations were initially amplified with the HLA-DQ- α primer pair to verify that the DNA was suitable for PCR analysis.

6.2.2 Amplification and cloning of the V3 loop fragment

Ten microlitres of the prepared DNA samples were amplified by PCR with nested primer pairs which flanked the V3 loop region of the *env* gene. The primers were designed by Dr C. Williamson from the Department of Clinical Virology, Medical School, UCT, and are described in Table 11. Degeneracies were included in the primers in order to select for both Western and African strains of HIV. Primer length was limited to between 18 and 25 nucleotides and degeneracies near the 3' ends were avoided. The expected amplification sizes for the outer and inner primer pairs were calculated at 424bp and 329bp respectively.

Table 11 Degenerate nested primers used for the amplification of the V3-region of HIV-1

ENV primer pairs	Sequence (5'-3')	Size of fragment
6545 outer forward	GCACAGTACAATGTACACATGG	424 bp
6944 outer reverse	*****C TAGAAAAATTCTCCTCTACAATTAA *****C***C	
6599 inner forward	TGAATGGCAGTCTAGCAGAAG	329 bp
6910 inner reverse	**A*****T****C TTCTGGGTCCCCTCCTGA AA*A	

*Bold letters illustrates where degeneracies may occur

The "standard" PCR protocol (chapter 3.1.1) was used for the amplification, except that, as mentioned before, the annealing temperature was changed to 43°C to accommodate the primers. Briefly, the samples were first amplified for 30 cycles with the outer primers (50pmol of each), then 5µl of the product from the first PCR was amplified for 40 cycles with the inner primers (50pmol). HIV-I negative DNA controls, blank reagent controls and positive patient DNA controls were included in each run.

6.2.2.1 Purification of PCR product

In order to obtain sufficient DNA, four separate PCR amplifications were performed for each patient and then PCR products were pooled. The excess of PCR primers was removed and the PCR products were concentrated by selective ammonium acetate precipitation [pCR-Script™ SK(+) cloning kit (Stratagene)]. A 1/10 volume of 10X STE buffer (1M NaCl, 100mM Tris.HCl, 10mM EDTA, pH8.0), an equal volume of 4M NH₄OAc, pH4.9 and 2,5 volumes of 100% (v/v) ethanol, at room temperature, were added to 100µl of the pooled product. The suspension was immediately centrifuged in a Beckman microfuge at room temperature for 20min at 8000 rpm. The DNA pellet was washed in 70%(v/v) ethanol, dried in a Speed Vac SC110 vacuum drier (Savant), resuspended in 20µl of TE-buffer (10mM Tris-HCl, 1mM EDTA, pH7.6) and stored at 4°C until used for cloning (section 6.2.2.2). The DNA concentration was estimated by comparing the intensity of the DNA band with that of a known standard (molecular weight marker II, Boehringer Mannheim) after electrophoresis on agarose gel and staining with EtBr.

6.2.2.2 Cloning of the purified product

The PCR amplified fragment of the *env* gene of HIV-I was cloned into the pBluescriptSK(+) phagemid (a 2958bp phagemid derived from pUC 19)

(Fig. 32) according to an adaptation of the method given in the manufacturer's instructions (Stratagene). Briefly, 10ng of pBluescript vector was ligated to 65ng of purified PCR fragment in the presence of 1X ligation buffer [25mM Tris.HCl pH7.5; 10mM MgCl₂; 10mM dithiothreitol; 20µg/ml bovine serum albumin (BSA) and 10mM rATP and 4U T4 DNA ligase, in a final reaction volume of 11µl. The restriction endonuclease EcoRV, was used in the ligation, instead of the Srf 1 recommended by the manufacturer. The ligation sample was mixed gently, incubated for one hour at room temperature, heated for 10min at 65°C and stored on ice until used for the transformation. A similar ligation, but omitting the PCR fragment, was prepared in parallel to assess the extent of vector self-ligation.

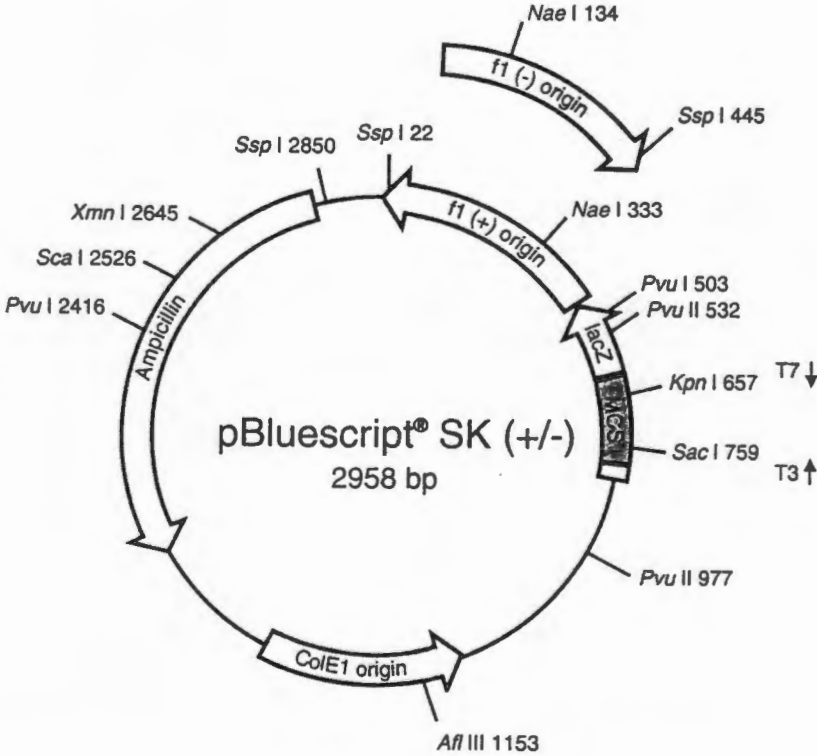


Fig. 32 Restriction endonuclease map of the cloning vector, pBluescriptSK(+) obtained from Stratagene.

The ligated plasmid was transformed into LK111 competent *Escherichia coli* cells (Dagert and Ehrlich, 1979). Two vials, each containing 100 μ l of competent cells, were thawed on ice, and 2 μ l of either the ligation mixture (with DNA) or the ligation control (without DNA) were added. After swirling the vials very gently, they were placed on ice for 30min, heat shocked at 42 $^{\circ}$ C for 60sec and placed on ice for 2min. Pre-warmed Luria broth (0.9ml) was then added to each vial and incubated for one hour at 37 $^{\circ}$ C with aeration at 225rpm in a Lab-line orbital shaker. The transformed cells were plated onto Luria agar containing 50mg/ml ampicillin, 20mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal substrate) [Boehringer Mannheim] and 200mg/ml isopropyl- β -D-thiogalactopyranoside (IPTG). Three hundred microlitres of each sample were plated onto separate agar plates and incubated at 37 $^{\circ}$ C overnight. Transformed colonies were identified by the blue/white colour selection technique of Sambrook *et al.* (1989). Bacteria containing plasmids with inserts appear as white colonies against a background of blue colonies lacking inserts. Insertion of DNA at this multiple cloning site interrupts the β -galactosidase (β -gal) gene and destroys its activity. This results in the appearance of white colonies when X-gal is supplied as the substrate. Cells containing plasmids without inserts express β -gal activity and catalyze the conversion of X-gal substrate into a blue pigment, thus giving rise to blue colonies.

Forty-six white colonies were selected, 25 from the mother and 21 from her infant. These colonies were re-plated onto duplicate X-gal-, IPTG- and ampicillin- containing Luria agar grid plates using a single sterile pipette tip per colony. The plates were incubated at 37 $^{\circ}$ C overnight. One of the grid plates contained a piece of nylon membrane (Hybond N, Amersham) that was later used for colony blot hybridization. The same tip as used to inoculate the grid plates was also used to inoculate the

bacteria into 2ml of Luria broth (containing 50mg/ml ampicillin). This was incubated overnight at 37°C with aeration (shaking at 200rpm in a Lab-line orbital shaker) and then used for small-scale preparations of plasmids.

6.2.3 Small scale plasmid preparations

A quick boiling method was used for the plasmid isolation (Berghammer and Auer, 1993). Aliquots of 1,5ml of an overnight culture were transferred into microfuge tubes (Eppendorf) and centrifuged in a Beckman microfuge E at 12000rpm for 30sec. The supernatant fluid was carefully removed by pipetting and the cell pellet resuspended in 30µl of lysis buffer [10mM Tris-HCl, pH8.0; 1mM EDTA; 15% (w/v) sucrose; 2mg/ml lysozyme; 0,2mg/ml pancreatic RNase and 0,1mg/ml BSA], and incubated with shaking at room temperature for 5min to allow the lysozyme to perforate the bacterial cell wall. The suspension was boiled for 60sec in a water bath and centrifuged in a Beckman microfuge E at 12000rpm for 20min at room temperature. Cell debris and genomic DNA formed a compact pellet, stabilized by the heat-precipitated BSA. The supernatant fluid was then used for restriction enzyme analysis. The restriction enzyme PvuII, which cleaves the vector at two sites, was used for the analysis. The smaller fragment contains the cloning cassette and, should there be an insert, this band will migrate at a slower rate than the same fragment from non-recombinant plasmids when subjected to agarose gel electrophoresis. Eight microlitres of DNA was mixed with 1µl of 10X Buffer M (Boehringer Mannheim) and 1µl PvuII, [2 units/microlitre] (Boehringer Mannheim), in a final volume of 10µl. The mixture was incubated at 37°C for 2hr and then heated at 65°C for 10min to inactivate the enzyme. The total 10µl volume of sample was then electrophoresed

in 2,5% agarose containing 0,1mg/ml EtBr (Merck). The stained DNA fragment was visualized on a UVP transilluminator.

6.2.4 Colony blot hybridization

The following procedure was used to liberate the DNA from the bacterial colonies and to bind it to the nylon membrane Hybond N (Amersham). Four pieces of Whatman 3MM paper were cut and fitted onto the bottoms of four glass petri dishes. Each piece was saturated with one of the following solutions: 10% SDS, denaturing solution, neutralizing solution, or 2X SSC (Appendix A). The nylon membrane was removed from the agar grid plate (chapter 6.2.2.2) after overnight incubation, and placed (colony side up) on the 10% SDS saturated filter paper, for 5min. This treatment limits the diffusion of the plasmid DNA during denaturation and neutralization, resulting in a sharper hybridization signal. After exposure to the SDS, the membrane was transferred to the second sheet of 3MM paper which had been saturated with denaturing solution. After a 10min exposure, the membrane was transferred to the third 3MM paper, saturated with neutralizing solution, and left for another 10min. After neutralization, the membrane was transferred to the last 3MM paper, which had been saturated with 2X SSC, and left for 5min. Finally, the membrane was also washed in 2X SSC by shaking at room temperature for 30min, and then dried, colony side up, on a sheet of 3MM paper. The DNA was then fixed to the nylon membrane by crosslinking, using a UV Crosslinker 1000 (Hoefer Scientific Instruments). This was followed by hybridization (see chapter 3.2.4.4) with a DIG-labelled probe. The PCR product from a PCR- and seropositive adult patient was labelled with digoxigenin for use as a probe, using the method described by Lo *et al.* (1990). Briefly, this method entailed using the "standard" PCR reaction, except that dTTP was replaced with a mixture of 0,065mM dTTP and 0,035mM DIG-dUTP (Boehringer Mannheim).

6.2.5 Large scale plasmid preparations

Eight positive clones (identified by colony blot hybridization) were selected for each patient (mother and infant) and large scale plasmid preparations were performed, using the Nucleobond AX PC kit (Macherey - Nagel, Germany) to purify the DNA. Each positive colony was inoculated into 100ml Luria broth, containing ampicillin, and incubated at 37°C overnight with shaking. The bacterial cells were concentrated by centrifugation at 6000rpm for 15min at 4°C in a Beckman J2-21 centrifuge, fitted with a JA-10 rotor. Cell disruption, isolation and purification of plasmid DNA was achieved using the Nucleobond AX PC kit (Macherey-Nagel, Germany), according to the manufacturer's instructions.

6.2.6 Sequencing of the cloned DNA fragments

Double-stranded plasmid DNA was sequenced by the dideoxy chain termination method (Sanger, 1977) using a T7 Sequencing kit (Pharmacia P-L Biochemicals). The DNA was denatured by mixing 10µl DNA (1,5 - 2,0µg of DNA) with 6µl of ultra-pure water and 4µl of 1M NaOH, and incubating the mixture at 37°C for 15min, whereafter it was placed on ice. The solution was neutralized by the addition of 8µl 3M sodium acetate (pH4.8). After the addition of 100µl of ice-cold absolute ethanol, DNA was precipitated by placing the tube at -70°C for 2hr. The precipitate was concentrated by centrifugation, washed with 70% ethanol, dried in a Speed-Vac vacuum drier and resuspended in 10µl of ultra-pure water. The denatured DNA was then mixed with 2µl of annealing buffer (T7 Sequencing kit) and 2µl of the universal forward or reverse primer (T7 Sequencing kit). The reaction mixture was incubated at 37°C for 30min in

a water bath, followed by 30min at room temperature and 4°C overnight or 5min at room temperature, to ensure primer annealing.

Labelling was achieved by adding 3µl of labelling mix (T7 Sequencing kit), 1µl of [α -³⁵S]-dATP (10µCi, Amersham) and 2µl of 1:5 diluted T7 DNA polymerase to the solution containing the annealed template and primer. This mixture was centrifuged briefly and then incubated for 5min at room temperature. The reaction was terminated by transferring 4,5µl of the sample into four pre-warmed dideoxynucleotide (ddATP, ddTTP, ddCTP, and ddGTP) tubes at 37°C for 5min. Termination solutions contained 4µM of one specific ddNTP as well as 40µM of all dNTPs in 50mM NaCl. After 5min incubation, 5µl of stop solution (T7 Sequencing kit) was added.

The principle of this method relies on the following: If a dideoxynucleotide triphosphate (ddNTP) is incorporated into a DNA strand, in place of dNTP, the chain cannot be extended further as ddNTPs contain no 3'-hydroxyl group. Terminations therefore occur specifically at each site of incorporation of ddNTP, e.g., if a primer and template are incubated with DNA polymerase in the presence of a mixture of ddTTP and dTTP, as well as the other three dNTPs, a mixture of fragments is obtained that all have dTTP 5' ends, and have ddTTP residues at their 3' ends. If this termination procedure is applied with all four nucleotide analogues separately, and the resulting fragments are electrophoresed side by side in adjacent lanes, a sequence ladder is produced which then can be used to determine the base sequence of the DNA.

A 6% denaturing acrylamide gel was used to separate the mixture of fragments. A 100ml working solution composed of 12ml of a 50% (w/v) bis-acrylamide solution (50% acrylamide 2,5% N,N'-bismethyleneacrylamide [NNB]), 10ml 10X NNB buffer (Appendix A), 48g urea and 84ml distilled water. The mixture of urea, NNB and water was

warmed to dissolve the urea, before the acrylamide was added. Polymerization of the acrylamide was initiated by the addition of 175 μ l of a 20% (w/v) ammonium persulphate solution and 40 μ l of TEMED (N,N,N',N'-tetramethylene-diamine). The liquid solution was slowly poured into the 0,4mm space between two glass plates of dimensions 40cm by 34cm, by lifting the plates to an angle of about 80°. At this angle the plates were only filled about 3/4 of the way, and they were then lowered, onto a rubber bung, to an angle of about 15° so that the acrylamide now filled the whole of the space. The straight edge of two shark's tooth combs (Hoefer Scientific Instruments) were inserted between the plates to provide a single, level well. The gel was allowed to set for at least one hour. If left overnight, the combs were covered with a piece of moist filter paper, and wrapped in clingwrap to prevent evaporation. The combs were removed, plates assembled in the electrophoresis tank (Omeg Scientific Instruments) and the gel warmed to 42°C by a pre-electrophoretic run for 30min at 35W (constant power) using 1X NNB in the lower tank and 0,6X NNB in the upper tank. The upper well was thoroughly rinsed with 0,6X NNB buffer to remove excess urea and the shark's tooth combs were then inserted into the well to create numerous evenly spaced partitions. The samples were heated to 78°C for 2min and 3 μ l of each sample was loaded into the wells. Electrophoresis was allowed to continue for either 3,5 or 5,5 hr at 70W, whereafter the buffer was removed from the upper buffer chamber and the top plate was slowly separated from the bottom plate by levering up one corner of the plate. The gel was transferred to 3MM Whatman paper, covered with clingwrap and dried on a SGD 4050 Slab gel dryer (Savant) at 80°C under vacuum for 2hr. The clingwrap was removed and the dried gel was subjected to autoradiography for one to five days. This entailed exposing the gel to Agfa Curix RPI X-ray film in the presence of a Cronex intensifying screen. The X-ray film was then developed as described in chapter 3.2.4.5.

6.2.7 Sequence analysis

Eight clones from the mother and five clones from her infant were sequenced. The V3 loop of all the clones was sequenced in both directions, whereas the variable region 3 *env* domain was only sequenced in one direction. The HIV sequences that were obtained from the mother and her infant were named according to the standardized sequence nomenclature proposed by the WHO (Korber *et al.*, 1994). The sequences of the mother's clones were named: 93ZA208.* (with * representing the 8 different clones, e.g. 93ZA208.i; 93ZA208.2; 93ZA208.W; 93ZA208.7; 93ZA208.P; 93ZA208.X; 93ZA208.1; 93ZA208.J) and the sequences of the infant's clones were named 93ZA209.* (* = X, 6, D, 5, 1). The first two characters refer to the year of isolation, the second two characters refer to the location where the sample was taken (ZA = South Africa) and the third three characters refer to the reference number for the study subject (208 = mother, 209 = infant) followed by *, indicating the name of the different clones.

Sequences were aligned using the computer program Clustal V (Higgins *et al.*, 1992), and the final alignment was done manually. The computer program TREECON (version 2.2) [Van den Peer and De Wachter, 1992] was employed for phylogenetic analysis, using the neighbour-joining approach and the distance matrix method (Kimura, 1980). Insertions/deletions were ignored and SIV_{CPZGAB} sequence was used as an outgroup. The validity of the branching pattern was assessed by performing 100 bootstrap analyses on the sequences. A branch point with a bootstrap value of >80% was considered reliable. The 13 datasets included a 347bp sequence (with gaps) and a 304bp sequence (without gaps) that were analyzed to draw the tree. Two representative reference sequences of each of subtypes A, B, C, and D of HIV-I were also included in the phylogenetic analysis.

The computer programs Genepro (version 6.1) were used to deduce the amino acid sequences of the V3 loop, and alignments were done using Clustal V (Higgins *et al.*, 1991; Higgins and Sharp, 1988).

6.3 Results

The mother and her infant were both sero-positive by HIV-I diagnostic criteria (chapter 4.2.2). As this infant was younger than 15 months old, the positive serology could have been attributed to maternal antibodies, and PCR (as described in chapter 5) was used to confirm his positive HIV status. DNA extracted from PBMCs from both mother and infant yielded positive HLA-DQ- α signals when amplified with the primers of this single copy gene. The 424bp product that was amplified by the outer V3 primers could not be visualized on an EtBr-stained agarose gel. After a nested amplification with the V3 inner primers, a 329bp fragment was visualized on an EtBr-stained agarose gel (Fig. 33).

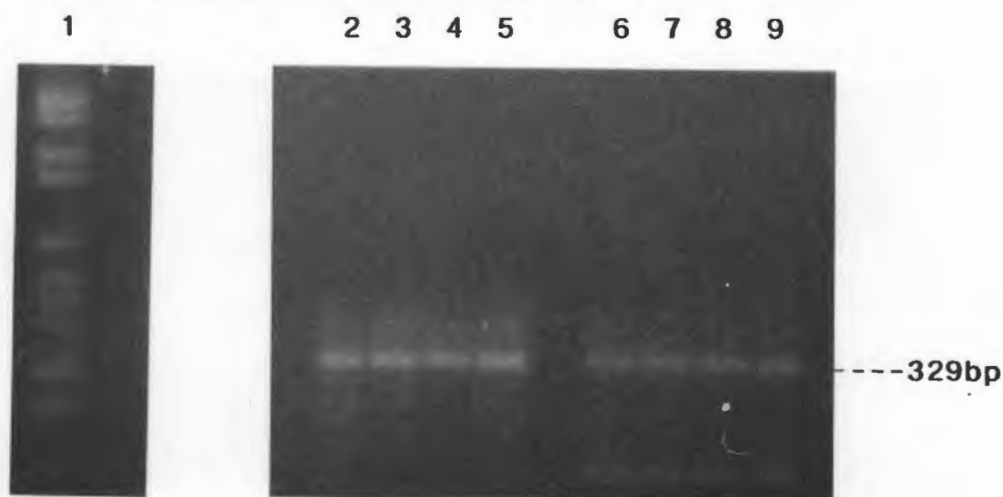


Fig. 33 Ethidium bromide stained agarose gel showing the 329bp fragment generated by the V3 inner primers. Lane 1, MWVI; lanes 2 to 5 amplification product of the infant; lanes 6 to 9 amplification products of the mother.

The 329bp fragment was cloned and positive clones were identified by colony blot hybridization [Figure 34(A) and (B)]. The clones are streaked in shape of alphabetical/numerical character for easy identification.

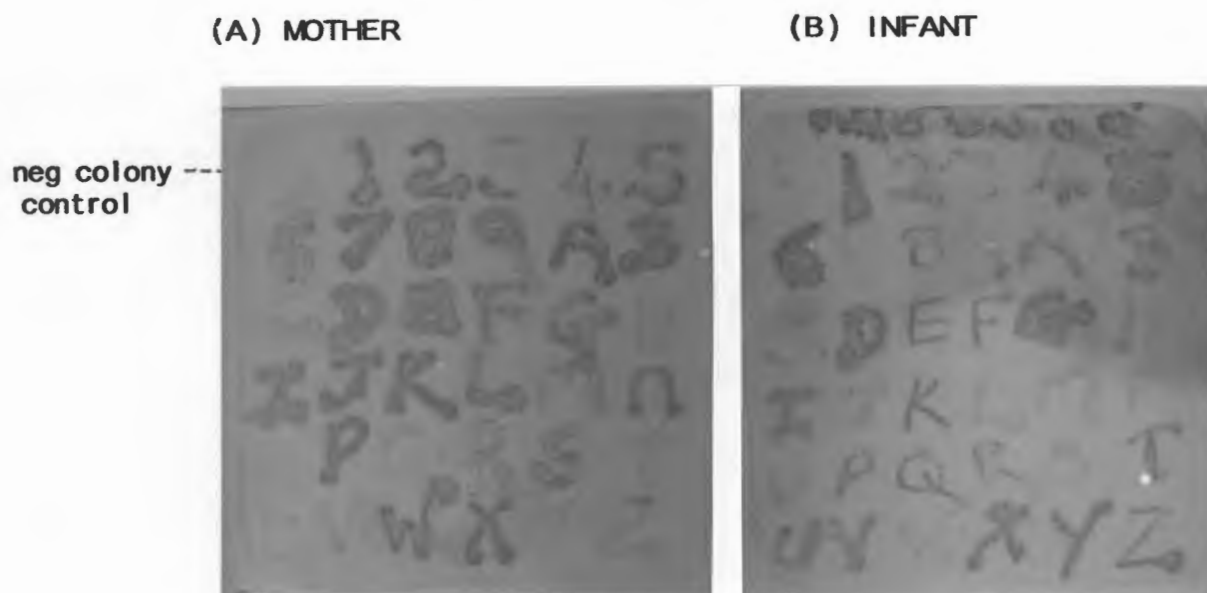


Fig. 34 Chemilumigram of the mother's clones (A) and her infant's clones (B) after colony blot hybridization. Clones were probed with a dig-labelled probe. Each clone are streaked in shape of an alphabetical/numerical character for easy identification.

Recombinant clones were not as readily detected by restriction enzyme analysis as by the colony blot hybridization (results not shown), and it was therefore decided to use the hybridization method as indicator of plasmids containing the cloned PCR fragment.

6.3.1 Sequence variation

Eight positive clones from the mother (2, P, W, 1, J, 7, I, X) and 5 positive clones from her infant (6, X, 1, D, 5) were selected by colony blot hybridization, and the DNA sequences were determined for each clone. The 329bp PCR fragment was sequenced (up to 320bp), and contained the immunodominant V3 loop. The computer-generated alignment of DNA sequences from both the mother and her infant are shown in figure 35, and a portion of the sequencing gels is illustrated in Fig. 36.

CLONE2	TAAATGGCAGCCTAGCAGAAGGAGACATAATAATCAAATCTGAAAATCTG	50
CLONEP	TAAATGGTAGCCTAGCAGAAGGAGACATAATAATTAAATCTGAAAATCTG	
CLONEW	TAAATGGCAGCCTAGCAGAAGGAGACATAATAATTAAATCTGAAAATCTG	
CLONEI	TAAATGGCAGCCTAGCAGAAGGAGACATAATAATCAAATCTGAAAATCTG	
CLONEJ	TAAATGGCAGCCTAGCAGAAGGAGACATAATAATTAAATCTGAAAATCTG	
CLONE7	TAAATGGTAGCCTAGCAGAAGGAGACATAATAATTAAATCTGAAAATCTG	
CLONEi	TAAATGGCAGCCTAGCAGAAGGAGACATAATAATCAAATCTGAAAATCTG	
CLONEX	TAAATGGCAGCCTAGCAGAAGGAGACATAATAATTAAATCTGAAAATCTG *****	
CLONE2	ACAAACAATGTCAAAACAATAATAGTACAGTTTAATGAATCTGTAGAAAT	100
CLONEP	ACAAACAATGTCAAAACAATAATAGTACAGTTTAATGAATCTGTAGAAAT	
CLONEW	ACAAACAATGTCAAAACAATAATAGTACAGTTTAATGAATCTGTAGAAAT	
CLONEI	ACAAACAATGTCAAAACAATAATAGTACAGTTTAATGAATCTGTAGAAAT	
CLONEJ	ACAAACAATGTCAAAACGATAATAGTACAGTTTAATGAATCTGTAGAAAT	
CLONE7	ACAAACAATGTCAAAACAATAATAGTACAGTTTAATGAATCTGTAGAAAT	
CLONEi	ACAAACAATGTCAAAACAATAATAGTACAGTTTAATGAATCTGTAGAAAT	
CLONEX	ACAAACAATGTCAAAACGATAATAGTACAGTTTAATGAATCTGTAGAAAT *****	
CLONE2	TGTGTGTATAAGACCCAACAACAATACAAGACAAAGTATAAGGATAGGAC	150
CLONEP	TGTGTGTATAAGACCCAACAACAATACAAGACAAAGTATAAGGATAGGAC	
CLONEW	TGTGTGTATAAGACCCAACAACAATACAAGACAAAGTATAAGGATAGGAC	
CLONEI	TGTGTGTATAAGACCCAACAACAATACAAGACAAAGTATAAGGATAGGAC	
CLONEJ	TGTGTGTATAAGACCCAACAACAATACAAGACAAAGTATAAGGATAGGAC	
CLONE7	TGTGTGTATAAGACCCAACAACAATACAAGACAAAGTATAAGGATAGGAC	
CLONEi	TGTGTGTATAAGACCCAACAACAATACAAGACAAAGTATAAGGATAGGAC	
CLONEX	TGTGTGTATAAGACCCAACAACAATACAAGACAAAGTATAAGGATAGGAC *****	
CLONE2	CAGGACAAACATTCTATGCAACAGGAGCCATAATAGGAGATATAAGACAA	200
CLONEP	CAGGACAAACATTCTATGCAACAGGAGCCATAATAGGAGATATAAGACAA	
CLONEW	CAGGACAAACATTCTATGCAACAGGAGCCATAATAGGAGATATAAGACAA	
CLONEI	CAGGACAAACATTCTATGCAACAGGAGCCATAATAGGAGATATAAGACAA	
CLONEJ	CAGGACAAACATTCTATGCAACAGGAGCCATAATAGGAGATATAAGACAA	
CLONE7	CAGGACAAACATTCTATGCAACAGGAGCCATAATAGGAGATATAATACAA	
CLONEi	CAGGACAAACATTCTATGCAACAGGAGCCATAATAGGAGATATAAGACAA	
CLONEX	CAGGACAAACATTCTATGCAACAGGAGCCATAATAGGAGATATAAGACAA *****	
CLONE2	GCACATTGTAACATTAGCGGAAAGAAGTGGAATGAACTTTAGAAAAAGT	250
CLONEP	GCACATTGTAACATTAGCGGAAAGAAGTGGAATGAACTTTAGAAAAAGT	
CLONEW	GCACATTGTAACATTAGCGGAAAGAAGTGGAATGAACTTTAGAAAAAGT	
CLONEI	GCACATTGTAACATTAGCGGAAAGAAGTGGAATGAACTTTAGAAAAAGT	
CLONEJ	GCACATTGTAACATTAGCGGAAAGAAGTGGAATGAACTTTAGAAAAAGT	
CLONE7	GCACATTGTAACATTAGCGGAAAGAAGTGGAATGAACTTTAGAAAAAGT	
CLONEi	GCACATTGTAACATTAGCGGAAAGAAGTGGAATGAACTTTAGAAAAAGT	
CLONEX	GCACATTGTAACATTAGCGGAAAGAAGTGGAATGAACTTTAGAAAAAGT *****	
CLONE2	AAGTATAAAATTAAAAGAACACTTCCCTAATAAAAACAATAAAATTTGCAC	300
CLONEP	AAGTATAAAATTAAAAGAACACTTCCCTAATAAAAACAATAAAATTTGCAC	
CLONEW	AAGTATAAAATTAAAAGAACACTTCCCTAATAAAAACAATAAAATTTGCAC	
CLONEI	AAGTATAAAATTAAAAGAACACTTCCCTAATAAAAACAATAAAATTTGCAC	
CLONEJ	AAGTATAAAATTAAAAGAACACTTCCCTAATAAAAACAATAAAATTTGCAC	
CLONEi	AAGTATAAAATTAAAAGAACACTTCCCTAATAAAAACAATAAAATTTGCAC	
CLONEX	AAGTATAAAATTAAAAGAACACTTCCCTAATAAAAACAATAAAATTTGCAC *****	
CLONE2	CACCCTCAGGAAGGGA	316
CLONEP	CACCCTCAGGAGGGGA	
CLONEW	CACCCTCAGGAAGGGA	
CLONEI	CAACCTCAGGAGGGGA	
CLONEJ	CACCCTCAGGAAGGGA	
CLONE7	CACCCTCAGGAAGGGA	
CLONEi	CA-CCTCAGGAGGGGA	
CLONEX	CA-CCTCAGGAGGGGA ** *****	

```

CLONE6 TAAATGGCAGCCTAGCAGAAGGAGACATAATAATTAAATCTGAAAATCTG 50
CLONEX TAAATGGTAGCCTAGCAGAAGGAGACATAATAATTAAATCTGAAAATCTG
CLONE1 TAAATGGCAGCCTAGCAGAAGGAGACATAATAATTAAATCTGAAAATCTG
CLONED TAAATGGTAGCCTAGCAGAAGGAGACATAATAATTAAATCTGAAAATCTG
CLONE5 TAAATGGCAGCCTAGCAGAAGGAGACATAATAATTAAATCTGAAAATCTG
*****

CLONE6 ACAAACAATGTCAAAACAATAATAGTACAGTTTAATGAATCTGTACAAAT 100
CLONEX ACAAACAATGTGTCAAAACAATAATAGTACAGTTTAATGAATCTGTAGAAGT
CLONE1 ACAAACAATGTGTCAAAACAATAATAGTACAGTTTAATGAATCTGTACAAAT
CLONED ACAAACAATGTGTCAAAACAATAATAGTACAGTTTAATGAATCTGTAGAAGT
CLONE5 ACAAACAATGTGTCAAAACAATAATAGTACAGTTTAATGAATCTGTAGAAGT
*****

CLONE6 TGTGTGTATAAGACCCAACAACAATACAAGACAAAGTATAAGGATAGGAC 150
CLONEX TGTGTGTATAAGACCCAACAACAATACAAGACAAAGTATAAGGATAGGAC
CLONE1 TGTGTGTATAAGACCCAACAACAATACAAGACAAAGTATAAGGATAGGAC
CLONED TGTGTGTATAAGACCCAACAACAATACAAGACAAAGTATAAGGATAGGAC
CLONE5 TGTGTGTATAAGACCCAACAACAATACAAGACAAAGTATAAGGATAGGAC
*****

CLONE6 CAGGACAAACATTCTATGCAACAGAGGCATAATAGGAAATATAAGACAAG 200
CLONEX CAGGAGCAACATTCTATGCAACAGAGGCATAATAGGAAATATAAGACAAG
CLONE1 CAGGACAAACATTCTATGCAACAGAGGCATAATAGGAA-TATAAGACAAG
CLONED CAGGACAAACATTCTATGCAACAGAGGCATAATAGGAAATATAAGACAAG
CLONE5 CAGGACAAACATTCTATGCAACAGAGGCATAATAGGAAATATAAGACAAG
*****

CLONE6 CATATTGTAACATTAGCGAAAAGAAGTGGAATAAAACTTTAGAAAAAGTA 250
CLONEX CATATTGTAACATTAGCGAAAAGAAGTGGAATAAAACTTTAGAAAAAGTA
CLONE1 CATATTGTAACATTAGCGAAAAGAAGTGGAATAAAACTTTAGAAAAAGTA
CLONED CATATTGTAACATTAGCGAAAAGAAGTGGAATAAAACTTTAGAAAAAGTA
CLONE5 CATATTGTAACATTAGCGAAAAGAAGTGGAATAAAACTTTAGAAAAAGTA
*****

CLONE6 AGTATAAAATTAAAAGAACAACCTCCCTAATAAAAACAATAAAATTTGCACC 300
CLONEX AGTATAAAATTAAAAGAACAACCTCCCTAATAAAAACAATAAAATTTGCACC
CLONE1 AGTATAAAATTAAAAGAACAACAACCCTAATAAAAACAATAAAATTTGCACC
CLONED AGTATAAAATTAAAAGAACAACAACCCTAATAAAAACAATAAAATTTGCACC
CLONE5 AGTATAAAATTAAAAGAACAACAACCCTAATAAAAACAATAAAATTTGCACC
*****

CLONE6 ACCTCAGGAGGGGACTCAGA 320
CLONEX ACCTCAGGAGGGGACTCAGA
CLONE1 ACCCTCAGGAGGGGACTCAGA
CLONED ACCCTCAGGAGGGGACTCAGA
CLONE5 ACCCTCAGGAGGGGACTCAGA
*** *

```

Fig. 35 DNA sequence alignment of the V3 region of HIV-1 as amplified from the PBMCs of the mother and her 3 month old baby. (A) shows the sequence alignment of the 8 positive clones obtained from the mother and (B) shows the sequence alignment of the 5 positive clones obtained from her infant.

Table 12 Percentage DNA distance and similarity in the V3 region. Genetic distances were calculated by the Kimura two-parameter method. Gaps in the alignment were ignored in the calculation.

CLONENVIRUS	% DIVERGENCE																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. 93ZA209.6	-	1.7	1.7	2.4	2.0	4.0	2.7	2.4	2.4	2.7	3.4	2.7	4.0	15.8	11.9	11.1	64.6
2. 93ZA209.X	98.3	-	3.4	2.7	3.1	4.1	3.1	3.4	3.4	3.7	3.7	3.7	4.1	16.2	12.2	10.7	65.1
3. 93ZA209.1	98.3	96.6	-	0.7	0.7	4.8	4.4	4.1	4.1	4.1	5.1	4.4	4.1	16.2	13.8	13.0	65.9
4. 93ZA209.D	97.6	97.3	99.3	-	0.3	4.8	3.7	4.1	4.1	4.1	4.4	4.4	4.1	15.4	13.0	12.8	66.1
5. 93ZA209.5	98.0	96.9	99.7	99.7	-	4.4	4.9	3.7	3.7	3.7	4.8	4.1	3.7	15.8	13.4	12.6	66.1
6. 93ZA208.2	96.0	95.9	95.2	95.2	95.6	-	1.0	0.7	0.6	1.7	1.0	0.3	1.3	5.8	10.7	9.9	64.7
7. 93ZA208.P	97.3	96.9	95.6	96.3	95.1	99.0	-	1.0	1.0	1.3	6.0	0.6	1.0	14.6	9.5	8.7	63.8
8. 93ZA208.W	97.6	96.6	95.9	95.9	96.3	99.3	99.0	-	0.6	1.0	1.0	1.0	1.3	14.9	9.9	9.1	65.6
9. 93ZA208.1	97.6	96.6	95.9	95.9	96.3	99.4	99.0	99.4	-	1.0	1.7	0.3	1.3	14.9	9.1	9.1	64.7
10. 93ZA208.J	97.3	96.3	95.9	95.9	96.3	98.3	98.7	99.0	99.0	-	2.0	1.3	0.6	14.9	10.3	9.5	64.6
11. 93ZA208.7	96.6	96.3	94.9	95.6	95.2	99.0	99.4	99.0	98.3	98.0	-	1.3	1.7	15.4	10.3	9.5	65.5
12. 93ZA208.1	97.3	96.3	95.6	95.6	95.9	99.7	99.4	99.0	99.7	98.7	98.7	-	1.0	15.4	10.3	9.5	63.8
13. 93ZA208.X	96.0	95.9	95.9	95.9	96.3	98.7	99.0	98.7	98.7	99.4	98.3	99.0	-	15.0	10.7	9.9	63.0
14. NOF(C)	94.2	83.8	83.8	84.6	84.2	84.2	85.4	85.1	85.1	95.1	84.6	84.6	85.0	-	13.3	14.1	63.4
15. 747(C)	88.1	87.8	86.2	87.0	86.8	89.3	90.5	90.1	90.9	89.7	89.7	89.7	89.3	86.7	-	4.1	63.8
16. 760(C)	88.9	89.3	87.0	87.8	87.4	90.1	91.3	90.9	90.9	90.5	90.5	90.5	90.1	85.9	95.9	-	65.9
17. SIV	35.4	34.9	34.1	33.9	33.9	35.3	36.2	34.4	35.3	35.4	34.5	36.2	37.0	36.6	36.2	34.1	-

% BASE SIMILARITY

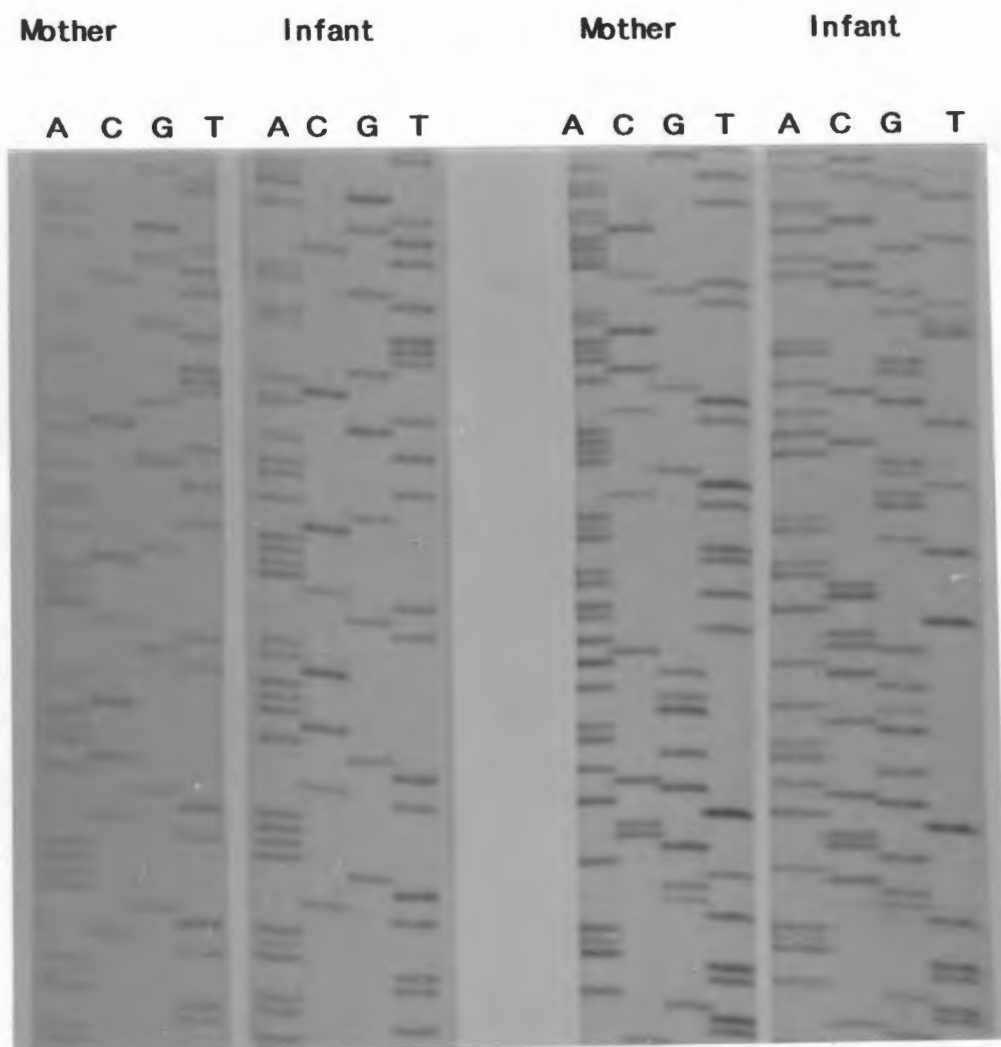


Fig. 36: Sequencing gels illustrate the base sequence of the V3 region of two clones from both mother and infant.

The population of HIV-I *env* sequences from the mother were largely homogeneous. Five of the 8 clones yielded identical sequences. In contrast, the infant's sequences were more divergent. Base similarity analysis between the consecutive pairs of viral sequences was determined and the values are listed in table 12 (page 158) in the form of a matrix. The mean intra-sample variation in sequences from the mother is 1,04% (range 0,6 to 2%). The infant's mean intra-sample variation is 1,8% (range 0,3 to 3,4%). Inter-patient sequence analysis between mother and baby showed a mean variation of 3,88% (range 2,4 to 5,1%).

A phylogenetic tree (Fig. 37) was generated using the aligned V3 sequences of HIV-I variants from the mother, her infant and previously

published subtypes: U455, Z321 representing subtype A; JRFL, LAI subtype B; NOF, D747, D760 subtype C; JYI, NDK subtype D; and SIV_{CPZ} (Myers, 1994). This tree showed that DNA sequences from both the mother and baby clustered with the HIV-I subtype C. They were, respectively, 86,4% and 87,5% similar to the sequences of other known subtype C viruses.

The predicted amino acid sequences of the V3 region of virus clones from the mother and her infant are shown in figure 38(A) and 38(B). In all cases an identical tetrapeptide motif, **GPGQ** (shown in bold in fig. 38), was present at the crown of the V3 loop. However, the same degree of identity did not extend across the entire octameric tip of the V3 loop: Whereas all variants isolated from the mother displayed an **RIGPGQTF** amino acid sequence, the predominant sequence in variants from the baby was found to be **RIGPGQAF**, indicating a substitution of threonine (T) with an alanine (A). Furthermore, both the mother's and the baby's sequences lacked a potential N-linked glycosylation site at the start of the V3 loop (as indicated in fig. 38). Valine (V) replaced asparagine (N) at this site in all the sequences.

Other substitutions were also observed within the V3 domain of variants from the infant. Clones D and 5 showed a threonine (T) at position 42 and glycine (G) at position 59, whereas clones 6, 1, and X all showed a serine (S) and alanine (A) respectively at these positions.

Distance 0.1

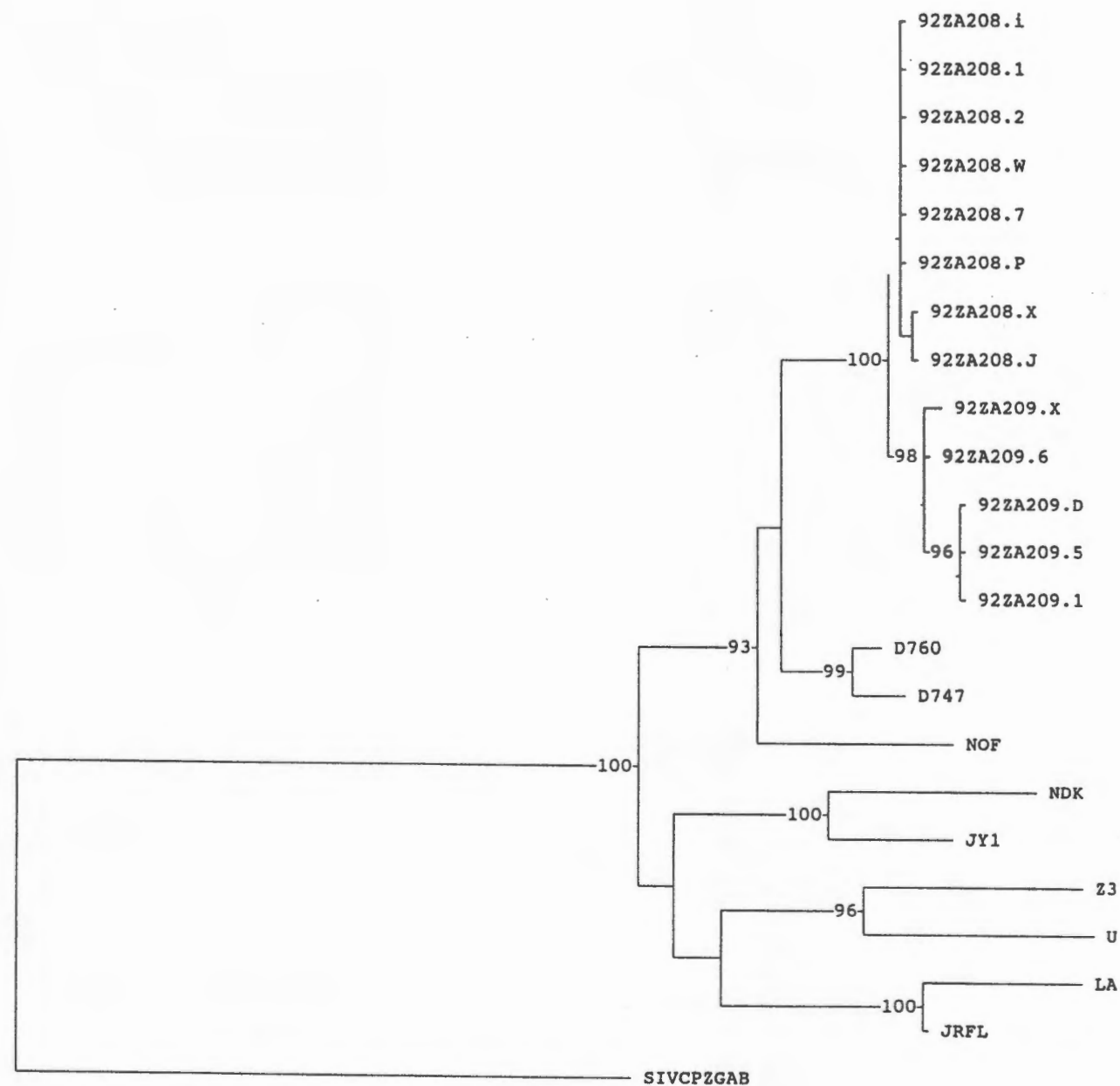


Fig. 37 Neighbour-joining phylogenetic tree resulting from 100 bootstrap replicates on the partial *env* sequence set. The tree was rooted on the SIV_{CPZGAB} sequence. Horizontal distances are proportional to sequence differences between species, vertical distances are arbitrary. Figures along the horizontal branches represent percentage bootstrap values greater than 95%. Clones derived from the mother and her baby are represented by 93ZA208 and 93ZA209 respectively.

		V3 loop	
		----->	
(A) Mother		*	
CLONE2	NGSLAEGDIIKSENLTNNVKTIIIVQFNESVEIVCIRPNNNTRQ SIRIGP		50
CLONEP	NGSLAEGDIIKSENLTNNVKTIIIVQFNESVEIVCIRPNNNTRQ SIRIGP		
CLONEW	NGSLAEGDIIKSENLTNNVKTIIIVQFNESVEIVCIRPNNNTRQ SIRIGP		
CLONEI	NGSLAEGDIIKSENLTNNVKTIIIVQFNESVEIVCIRPNNNTRQ SIRIGP		
CLONEJ	NGSLAEGDIIKSENLTNNVKTIIIVQFNESVEIVCIRPNNNTRQ SIRIGP		
CLONE7	NGSLAEGDIIKSENLTNNVKTIIIVQFNESVEIVCIRPNNNTRQ SIRIGP		
CLONEi	NGSLAEGDIIKSENLTNNVKTIIIVQFNESVEIVCIRPNNNTRQ SIRIGP		
CLONEX	NGSLAEGDIIKSENLTNNVKTIIIVQFNESVEIVCIRPNNNTRQ SIRIGP		

		<-----	
CLONE2	GQTFYATGAIIGDIRQAHCNISGKKWNETLEKVS IKLKEHFPNKT IKFAP		100
CLONEP	GQTFYATGAIIGDIRQAHCNISGKKWNETLEKVS IKLKEHFPNKT IKFAP		
CLONEW	GQTFYATGAIIGDIRQAHCNISEKKWNETLEKVS IKLKEHFPNKT IKFAP		
CLONEI	GQTFYATGAIIGDIRQAHCNISEKKWNETLEKVS IKLKEHFPNKT IKFAP		
CLONEJ	GQTFYATGAIIGDIRQAHCNISEKKWNETLEKVS IKLKEHFPNKT IKFAP		
CLONE7	GQTFYATGAIIGDIQAHCNISGKKWNETLEKVS IKLKEHFPNKT IKFAP		
CLONEi	GQTFYATGAIIGDIRQAHCNISGKKWNETLEKVS IKLKEHFPNKT IKFAP		
CLONEX	GQTFYATGAIIGDIRQAHCNISGKKWNETLEXV SIKLKEHFPNKT IKFAP		

CLONE2	PSGRDS		106
CLONEP	PSGGDP		
CLONEW	PSGRDS		
CLONEI	TSGGDS		
CLONEJ	PSGRGR		
CLONE7	PSGRDP		
CLONEi	PQEGTK		
CLONEX	PQEG--		
		V3 loop	
		----->	
(B) Infant		*	
CLONE6	NGSLAEGDIIKSENLTNNVKTIIIVQFNESVQIVCIRPNNNTRQ SIRIGP		50
CLONE1	NGSLAEGDIIKSENLTNNVKTIIIVQFNESVQIVCIRPNNNTRQ SIRIGP		
CLONED	NGSLAEGDIIKSENLTNNVKTIIIVQFNESVEIVCIRPNNNSRQ SIRIGP		
CLONE5	NGSLAEGDIIKSENLTNNVKTIIIVQFNESVEIVCIRPNNNSRQ SIRIGP		
CLONEX	NGSLAEGDIIKSENLTNNVKTIIIVQFNESVEVVCIRPNNNTRQ SIRIGP		

		<-----	
CLONE6	GQAFYATGGIIGNIRQAYCNISEKKWNKTLEKVS IKLKEHFPNKT IKFAP		100
CLONE1	GQTFYATGGIIGDIRQAYCNISEKKWNKTLEKVS IKLKEHFPNKT IKFAP		
CLONED	GQAFYATGAIIGNIRQAYCNISEKKWNKTLEKVS IKLKEHNP PNKT IKFAP		
CLONE5	GQTFYATGAIIGNIRQAYCNISEKKWNKTLEKVS IKLKEHNP PNKT IKFAP		
CLONEX	GQAFYATGGIIGNIRQAYCNISEKKWNKTLEKVS IKLKEHFPNKT IKFAP		
		*,*****,***,*****	
CLONE6	PSGGDS		106
CLONE1	PSGGDS		
CLONED	PSGGTQ		
CLONE5	PSGGTQ		
CLONEX	PSGGDS		

Fig. 38 The deduced amino acid sequences of the V3 loop region of HIV-I clones from the mother (A) and her infant (B). The valine which replaces a potential N-linked glycosylation site is marked with an asterisk (*). The tetrameric tip is shown in bold letters, and the V3 loop is indicated by the arrowed line.

6.4 Discussion

Phylogenetic analysis of HIV-I viruses isolated from both the mother and her infant in this study, have placed them in the same cluster as the other known HIV-I subtype C viruses. To date, forty isolates of HIV-I subtype C have been sequenced, of which 38 have been published (Myers, 1994). Amongst these is the first described South African isolate of HIV-I (NOF) [Dietrich *et al.*, 1992] as well as numerous isolates from India and Zambia (Myers, 1992).

Data obtained from the analysis of the V3-loop of viral isolates from the mother/infant pair in this study is consistent with their subtype C classification: e.g. the absence of a potential N-linked glycosylation site proximal to the first cysteine of the V3 loop. This glycosylation site is commonly present in all HIV-I sequences except those of subtype C (Myers, 1994; Orloff *et al.*, 1993). The sequence of the octameric tip found in the V3 loop of our mother-infant pair, is also consistent with subtype C classification. The most common motif found in subtype C is, **RIGPGQTF**, but other subtypes differ in that **HIGPGQAF** is characteristic of subtype A, **HIGPGRAF** of subtype B, **PIGPGQAL** of subtype D, **TIGPGQVF** of subtype E and **HIGPGQAF** is characteristic of subtype F. It has been shown that amino acid changes near the center of this tip can influence the specificity of anti-V3 neutralizing antibodies (Myers, 1994). The central V3 tetrapeptide motif, **GPGQ**, was present in all the sequences from both mother and baby, and thus correlates with the findings that this motif is seen among HIV-I sequences of subtypes A, C, D, and E, in contrast to the **GPGR** motif that is commonly seen in subtype B. It's thought that this tip has a specific conformation (it forms a turn) that is important for neutralizing antibody epitopes as well as T-cell epitopes (Myers, 1994).

Information regarding the degree of similarity between variants from the mother and infant in this study, differs to a large extent from previous studies. We found that the virus population harboured by the mother displayed highly homogeneous V3 sequences (1,04% variation) compared to virus isolates from her 3 month old infant, which showed a higher degree (1,8%) of heterogeneity. Most other studies have indicated that there is a trend towards higher variability with time, and generally, sequences of isolates from mothers were far more heterogeneous than those of isolates from their infants. For example, Wike *et al.* (1992) examined the sequence diversity of HIV-1 between 4 maternal-infant transmission pairs, of which 3 were from the United States and the fourth from Rwanda. Isolates from the mothers in their study showed extensive diversity (5% in the V3 region) while the infants' isolates were more highly conserved with a mean distance of 2,5% (V3 region) between sequences. No clinical data or ages were given for these infants. In a similar study, comparing the variation in a mother and her child following transmission, Mulder-Kampinga *et al.* (1993) observed a heterogeneous sequence population in the maternal samples that were taken at 6, 4, and 2 months prior to delivery and 10 months after child birth, with a mean nucleotide variation (V3 region) of 2,4% to 4,2%. The sequence population of the infant was highly homogeneous until the age of 6 weeks (<0,7%) and only showed more heterogeneity at 9 months of age (1,8%). These authors suggested intra-uterine transmission based on their findings that the homogeneous sequence population in the child at birth was more closely related to the sequence population of the mother during the first and second trimester of pregnancy than to the sequence population at delivery and 10 months after delivery.

A third group of researchers, Wolinsky *et al.* (1992) analyzed different regions from the *env* gene (including the V3 region) from 3 mothers and their 2-4 month old babies. The viral sequences from the mothers in their study also showed a greater variability than that of their infants', who

showed a narrow distribution of variants. Scarlatti *et al* (1993) compared sequences from the V3 region from 10 mother-infant pairs. The mothers were all drug users or sexual partners of drug users. Nine of the 10 infants were shown to be asymptomatic and one child was reported to have hepatitis and hepatosplenomegaly. Similar results regarding the extensive heterogeneity of the maternal samples and homogeneous infant isolates were shown in their study. The reasons for the discrepancy between our findings and those of others are not clear. It is conceivable that the apparent homogeneity of the mother's variants was not a true reflection of her entire virus population. It could have been biased by selective amplification following low virus yield in the original blood specimen. An indication of this possibly occurring was given by the fact that the original PCR band obtained from the mother's blood sample was fainter than that of the baby's. Nevertheless, other reasons must be sought to explain the relative diversity of the variants from the infant. Although the 1,8% of heterogeneity which were observed was considerably higher than that of the mother, it was not as high as the 2,5% diversity observed in vertically infected infants by Wike *et al*. (1992).

The degree of nucleotide variation found in our 3 month old infant was only attained in the infant described in the study by Mulder-Kampinga *et al*. (1993), at 9 months of age. It may be relevant that the 9 month old infant was described as clinically asymptomatic with immunological disorders, whereas our infant had pneumonia and a week later his symptoms were described as "full blown AIDS". It is possible that the more rapid onset of disease might be related to the higher nucleotide variation in the younger infant. The early and rapid onset of disease in neonates is not uncommon, and seropositive infants as young as one month of age have been seen to manifest AIDS symptoms (Davachi, 1994). It has been suggested that a rapid progression is more pronounced in African infants, than in infants from the West. The nature

of the selective pressures are unknown, but it is thought that nutritional status may play a role. Semba *et al.* (1994) suggests a strong association between maternal vitamin A deficiency and an increase in vertical transmission of HIV-I.

Fouchier *et al.* (1995) investigated the role that phenotypic variability of HIV-I plays in the pathogenesis of AIDS. They suggested that a rapid decline of CD4 T-cells, a reduced survival time after AIDS diagnosis and a rapid disease progression are associated with the presence of syncytium-inducing (SI) HIV-I isolates. They showed that the presence of positively charged amino acid residues at position 306 and/or 320 in the V3 loop could be associated with the SI capacity of HIV-I (Fig. 39).

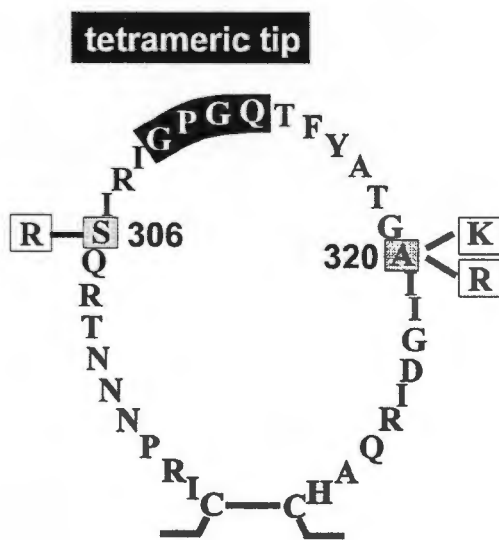


Fig. 39 Diagrammatic presentation of the amino acid sequence variation in V3 regions from syncytium- and non-syncytium-inducing (NSI) HIV-I isolates. Arginine (R) at position 306 in the V3 loop and/or arginine or lysine (K) at position 320 are associated with syncytium inducing isolates (Adapted from Fouchier, 1995).

De Wolf *et al.* (1994) examined SI- and NSI-associated V3 mutations in HIV-I subtypes other than subtype B. The cytopathic abilities of the different variants were predicted, based on the V3 loop amino acid sequences, assuming that positively charged amino acid substitutions at position 306 and 320 in the V3 loop is related to T-cell line tropic, SI-viruses, and that uncharged or negatively charged amino acid substitutions at these respective positions correlated with macrophage-

tropic, NSI-variants. On comparing their culture results with their V3 sequence results, they found a positive correlation for 28 of the 29 NSI virus stock samples, and 10 of the 12 SI virus stock samples, thus confirming that this type of analysis could be used for the prediction of biological phenotypes, not only for subtype B variants, but also for subtype A, C, D, and E variants. They concluded that prediction of disease progression could be made by determining the SI capacities of HIV-I isolates.

The application of the above predictive criteria (based on the amino acids present at positions 306 and 320 of the V3 loop) indicates that isolates from both the mother and her infant in our study, are consistent with the NSI-phenotype (Fig. 40(A), 40(B)).

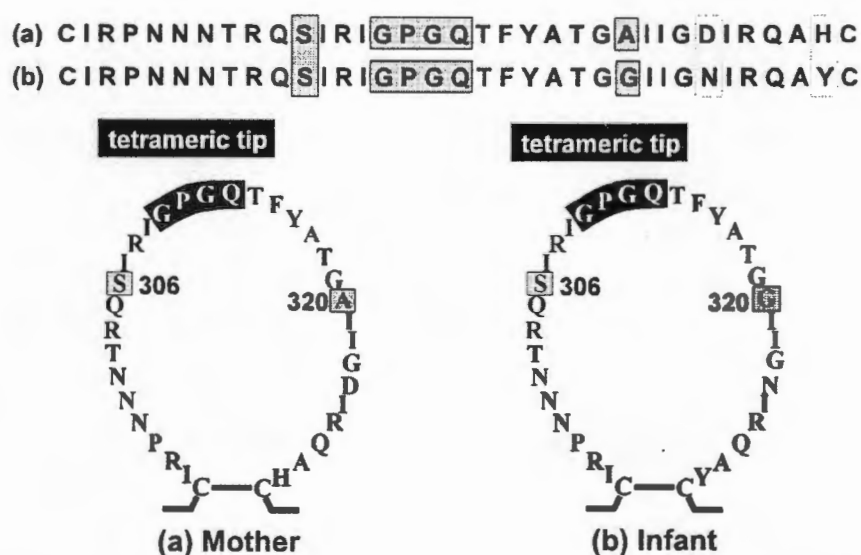


Fig. 40 Schematic presentation of the amino acid sequence variation in the V3 loop from the mothers' isolates (a) and her infants' isolates (b). The absence of positively charged amino-acids at positions 306 and 320, in both cases, indicates that the isolates are of the NSI variety.

From available sequence data, it appears that a mutation at position 306 is not commonly found in subtype C isolates. A comparison of 38 published subtype C sequences (Myers, 1994), reveals only one isolate (ZA.NOF) with a substitution at position 306, where a serine (S) residue

is substituted for an arginine (R) residue (indicative of SI phenotype). No substitution is noted at position 320 for this isolate.

Conflicting evidence thus arises from the different studies: the 3 month old infant in our study, diagnosed with “full blown AIDS”, was shown to harbour a NSI HIV-I isolate, despite the findings of Fouchier *et al.* (1995) that a rapid disease progression is associated with the presence of a SI HIV-I isolate. However, Saag *et al.* (1994) reported that SI variants are not required for HIV disease progression, and that only half of AIDS patients harbour SI variants, indicating that NSI HIV variants can also cause AIDS and death. Although varying opinions exists, it is necessary to bear in mind that factors such as the moment and manner of sampling, choice of primers and possible selection by amplification, virus load, and stage of disease may all variably influence the outcome of genetic epidemiological studies.

This type of study has not been done before in the local community on HIV-I subtype C isolates, and data presented here should be useful for future comparative studies.

Conclusions

One of the main achievements of this study was to devise, optimise and instigate a PCR assay for resolving the infection status of babies born to HIV-infected mothers, in the neonatal period. In order to achieve this, it was necessary to adapt and optimise a standard PCR protocol to ensure maximum sensitivity and specificity for HIV proviral DNA, and then to ensure that the assay was accurate and reproducible before it could be applied in our routine diagnostic laboratory.

Genetic variation is a hallmark of HIV-I, -II, and in the light of this variability, appropriate choice of primers in a diagnostic PCR assay is a factor of major importance. Consequently, different primers directed against various conserved regions in the HIV-I genome were used to ensure detection of all variants. A standard PCR protocol was optimised for all the sets of primers (SK29,30; SK145,431; SK38,39; SK68,69 nested) used during this study. In general this required an increase in the concentrations of $MgCl_2$, *Taq* DNA polymerase and dUTP, to give best performance with the primers of choice. The four sets of primers that were selected all performed equally well. Using them in various combinations in duplicate tests on 138 HIV seropositive sera (selected at random from the routine diagnostic laboratory), we were able to show 100% correlation between their respective PCR results. For the initial optimization reactions a plasmid, pBH10, that contains truncated HIV sequences, was chosen as template. As this plasmid-PCR system generates high copy numbers of HIV sequences, contamination was a very high risk that became an unfortunate reality. A great deal of time and effort was spent in an attempt to eliminate the problem, but without success. Finally, there seemed no alternative but to discard the contaminated primer pair involved (SK68,69) from the study, and a valuable lesson was learned regarding the need for meticulous attention to methods of containment.

As a precautionary measure, a dUTP-enzymatic system was incorporated into our HIV PCR assay. This incorporation led to more optimization experiments as the optimum concentration of dUTP in the PCR mixture differed from the previously established dTTP concentration. Theoretically, dU-containing fragments will be degraded by the UNG enzyme with no effect on the specific dT-containing template, but in our hands, the incorporation of the UNG-enzyme into the system resulted in severe inhibition of all amplification. Nevertheless, it was decided to maintain the incorporation of dUTP in all our HIV PCR reactions in order to generate dU-containing amplification products, so that in the event of unforeseen contamination, the UNG enzyme could be used in a broad program of eradication to clear all contaminants.

A major requirement of diagnostic assays applied to detection of infection in very young babies, is high sensitivity, since the volume of blood samples is invariably small. Our optimized PCR system compared very favourably with other published systems, and was able to detect as little as one copy of plasmid DNA. Specificity is another prime consideration in HIV diagnosis. It was established that a good correlation existed between the optimised HIV PCR assay and currently used 3rd generation ELISA assays:- The eleven seronegative patients were PCR negative and 83% (115/138) of the seropositive patients were also shown to be HIV PCR positive. Analysis of patient data revealed that the remaining 17% (23/138) that were negative by PCR, were all infants less than 15 months of age and their positive HIV sero-status was most probably a reflection of acquired maternal antibodies from their seropositive mothers. Taking all these results into consideration, it was concluded that the correlation between the HIV PCR and antibody results for older patients was close to 100%. This was very encouraging as it indicated that our HIV PCR system could identify all the HIV-I subtypes that were detectable by ELISA in our local community.

A number of important technical considerations were made apparent while performing the PCR assay on human DNA specimens, and they include: 1) Whole blood specimens should be processed as soon as possible after collection, as long periods of storage (24 - 48hr) at room temperature or 4°C can affect the yield of amplifiable DNA; 2) the specificity of the assay could be further increased by a combination of a temperature of 42°C, with a low salt concentration in the washing buffer (0,1X SSC) during the washes after oligonucleotide hybridization; 3) Adequate washing procedures during the extraction process that releases DNA from PBMCs are essential as haeme-related compounds can inhibit amplification; 4) Sufficient positive and negative controls should be included in each PCR run and HIV PCR results should be confirmed with specific probes by Southern blot hybridization since contamination and amplification of non-specific "back-ground" DNA sequences can pose potential problems in the interpretation of results; and 5) A constant vigilance is required to avoid the introduction of new sources of contamination.

Since a prime objective for the newly devised PCR assay was accurate diagnosis of HIV infection in very young babies, its performance was evaluated using blood specimens from 45 infants under 15 months of age, all born to HIV seropositive mothers, and for whom serology results were therefore inadequate to determine true infection status. The 3 infants who were seronegative were also PCR negative but HIV proviral DNA was detected in the PBMCs of 27 (64%) of the 42 seropositive infants. The positive PCR tests correlated well with the clinical data indicative of active HIV-infection for the majority of these infants. The remaining 15 (36%) seropositive infants were PCR negative, which, on the basis of the established high level of sensitivity and specificity of the assay, was strongly indicative of the absence of infection. We could, however, not exclude an HIV infection in all the babies who were very young (under 4 - 6 weeks of age) as low levels of virus could be present in peripheral blood or the virus could be confined in lymphoid organs at this young age (Krivine *et al.*,

1990). This also reduces the number of infected lymphocytes in the small samples of blood that can be taken from very young babies. In infants older than 3 months, one could predict with greater confidence that a negative PCR result probably indicates the absence of active infection. An additional application for a sensitive and specific PCR assay is to assist in the interpretation of the so-called "indeterminate" serological findings. It was decided that in spite of the high cost of the HIV PCR assay, the overall benefit of an early diagnosis may be sufficient to warrant its being put to more widespread use.

The same PCR protocol was also applied in the second part of this study to amplify HIV V3-specific sequences from an HIV seropositive, clinically well mother and her 3 month old infant, diagnosed with full blown AIDS. These DNA fragments were then cloned and sequenced (8 clones from the mother and 6 from her infant). This examination was stimulated by the increasing amount of conflicting data on the genetic variants involved in maternal-fetal transmission.

HIV is known to evolve within an individual during the course of infection resulting in nucleotide sequence variation within that individual. Examination of the V3-region in the *env* gene of HIV-I isolates from our mother-infant pair confirmed this variation. It was found that the virus population harboured by the mother was highly homogeneous (only 1,04% variation) compared to the virus population of her infant which showed a higher degree (1,8%) of heterogeneity. Regarding the degree of variation, our data differed from other studies where an extensive heterogeneity was shown in maternal samples and more homogeneity in infant samples. New data from this study, although obtained from only a single mother-infant pair, should add to the pool of information concerning variants involved in materno-fetal transmission and their rate of evolution, and suggests that fixed rules do not apply. Each investigation will undoubtedly be influenced by the clinical status of the patients at the time of sampling (stage of

disease, duration of infection, virus load, etc.) as well as the varying parameters of the respective technical protocols and the degree of selective amplification. These factors could in part account for the range of divergent opinions from different groups of researchers. Other conflicting evidence from this study includes the finding that the 3 month old infant (in whom AIDS was already well advanced) harboured only a non-syncytium-inducing type of HIV-I, despite the findings of others that a rapid disease progression is associated with the presence of syncytium-inducing HIV variants. It is reasonable to assume that the probability of transmission from mother to infant, as well as the rate of deterioration in the immune function, is dependent on many factors, and we conclude that the presence or absence of a SI-virus is insufficient to be used as a sole prognostic marker.

Phylogenetic analysis of the HIV-I viruses isolated from both mother and her infant have placed them in the same cluster as other known subtype C viruses. This classification was substantiated by more detailed genetic analysis of the V3 loop, which indicated the absence of a potential glycosylation site proximal to the first cysteine residue of the V3 loop, and a GPGQ central tetrapeptide motif of the V3 loop, characteristic of subtype C viruses. This provides further confirmation to other published data that subtype C viruses are present in South Africa.

In summary, this study has resulted in the development of a sensitive and specific PCR assay which will be (and is already being) used for HIV diagnostic purposes, particularly in the case of infants. It has also been shown that this technique can be effectively applied to a variety of genetic epidemiological studies. Since there is at present little information available on subtype C viruses involved in materno-fetal transmission in South Africa, data arising from this study should be useful for future comparative studies. It is hoped that the work presented in this thesis will make a significant contribution to the overall perspective of HIV and AIDS-related infections in this country.

Appendix A

Solutions and Buffers

50% (w/v) Acrylamide and 2,5% (w/v) N,N'-bismethyleneacrylamide

250g acrylamide

12,5g N,N'-bismethyleneacrylamide

Dissolve in distilled water and adjust volume to 500ml. Filter and store at 4°C in a dark bottle.

Ampicillin (100mg/ml)

2g Ampicillin

Dissolve in 20ml distilled water, aliquot and store at -20°C.

Buffer 1 for Chemiluminescent detection

0,1M maleic acid

0,15M NaCl

pH7.5

Blocking stock solution

10% solution in distilled water. Autoclave and store at -20°C.

Buffer 2 for Chemiluminescent detection

Blocking stock solution diluted 1:10 in Buffer 1

Final concentration = 1% blocking solution (Boehringer Mannheim)

Wash buffer for Chemiluminescent detection

Buffer 1 and 0,3% (w/v) Tween-20

Buffer 3 for Chemiluminescent detection

0,1M Tris.HCl

0,1M NaCl

50mM MgCl₂

pH9.5

Denaturing buffer for Southern blot

200g NaOH (0,5M)

876,6g NaCl (1,5M)

Make up to 10 l with distilled water

Ethidium bromide (EtBr) stock solution

1g EtBr

Dissolve in 100ml distilled water (wearing a mask). Stir for 1hr on a magnetic stirrer and store in a dark bottle at room temperature.

IPTG (200mg/ml)

0,2g IPTG

Dissolve in 1ml distilled water, aliquot and store at -20°C.

K buffer

50mM KCl

10mM Tris.HCl

2,5mM MgCl₂, pH8.3

100μg/ml fresh proteinase K

0,5% Tween 20

Lithium chloride (4M)

42,39g lithium chloride

Dissolve the lithium chloride in 250ml of distilled water and sterilize by autoclaving. Store the solution at room temperature.

Luria agar

10g tryptone

5g yeast extract

5g NaCl

15g agar

Dissolve the agar in 500ml of distilled water and dissolve the tryptone, yeast extract and NaCl in a separate 300ml aliquot. Mix the two solutions and increase the volume to 1l. Dispense the solution into bottles and sterilize by autoclaving at 15psi for 15min. Store at room temperature.

Luria broth

10g tryptone

5g yeast extract

10g NaCl

Dissolve the above components in distilled water and make up to 1l. Dispense the solution into bottles and sterilize by autoclaving at 15psi for 15min. Store at room temperature.

Lysis buffer for small scale plasmid preparations

10mM Tris.HCl

1mM EDTA

15% (w/v) sucrose

2mg/ml lysozyme

0,2mg/ml RNase

0,1mg/ml bovine serum albumin

Aliquot and store at -20°C

Neutralizing buffer for Southern blot

8,0g NaOH (20mM)

770,8g Ammonium acetate (1M)

make up to 10 l with distilled water

10X NNB buffer

162,0g Tris base

27,5g Boric acid

9,3g EDTA

Make up to 1000ml with distilled water.

Proteinase K (Boehringer Mannheim)

Dissolved at 20mg/ml in 10mM Tris.HCl (pH7.5)

20X SSC buffer

175,3g NaCl

88,2g Tri-sodium citrate

Dissolve the components in 800ml distilled water and adjust the pH to 7.0 with a small volume of 10M NaOH. Increase the volume to 1000ml with distilled water. Sterilize by autoclaving at 15psi for 15min and store at room temperature.

10% SDS

100g Sodium dodecyl sulphate

Dissolve in 900ml of distilled water and heat to 68°C to assist dissolution. Adjust the pH to 7.2 and make up to 1000ml with distilled water. Store at room temperature.

25% Sucrose

25g sucrose

1M TE buffer (pH 9.0)

Dissolve the sucrose in TE buffer and then make up to 100ml with the TE buffer. Sterilize the solution by autoclaving at 10psi for 10min and store at room temperature.

10X STE buffer

1M NaCl

100mM Tris.HCl

10mM EDTA

pH8.0

10X Taq PCR buffer

100mM Tris.HCl

15mM MgCl₂

500mM KCl

1mg/ml gelatine, pH8.3 at 20°C, Boehringer Mannheim Biochemica

50X Tris-acetate buffer (TAE)

242g Tris.HCl

57,1ml glacial acetic acid

100ml 0,5M EDTA pH8.0

Make up to 1000ml with distilled water and autoclave at 15 psi for 15min.

Tris-EDTA buffer (TE)

10mM Tris.HCl

1mM EDTA, pH7.6

Triton/doc solution

4,0ml TritonX-100

0,4g Sodium deoxycholate

1,0ml Tris.HCl (pH8.0)

0,2ml of 0,5M EDTA (pH8.0)

Dissolve the above constituents in 80ml distilled water, then adjust the volume to 100ml. Sterilize the solution by autoclaving at 15psi for 15min and store at room temperature.

X-gal (20mg/ml)

0,1g X-gal

Dissolve in 5ml dimethylformamide and store at 4°C.

Appendix B

Nucleotide code - single letters

A	adenosine triphosphate
C	cytidine triphosphate
G	guanosine triphosphate
T	thymidine triphosphate
U	uridine triphosphate

Amino acid code - single letters

A	alanine
R	arginine
N	asparagine
D	aspartic acid
C	cysteine
Q	glutamine
E	glutamic acid
G	glycine
H	histidine
I	isoleucine
L	leucine
K	lysine
M	methionine
F	phenylalanine
P	proline
S	serine
T	threonine
W	tryptophan
Y	tyrosine
V	valine

Appendix C

Photography

Black and white negatives of the chemilumigrams and Polaroid photographs were taken with a 35mm Leica DBP (Ernst Leitz, Germany) using an Ilford HP5 ASA 400 black and white film. The prints were made using an Opemus 5a enlarger fitted with a no. 3.5 filter from the Ilford multigrade filter set, and exposing them to Ilford multigrade III glossy photographic paper (England).

The prints were developed by a 2 to 3min immersion in Ilford paper developer (Ilfospeed) diluted 1:9 with water. They were then transferred into 2% acetic acid that acted as a stop solution and lastly fixed by immersion in Super Amfix (Champion) high speed fixer for paper (1:9 dilution), for 5-10min. The prints were washed for 10min in running water and dried in a warm air dryer.

The colour photographs were taken with the same 35mm camera using Fuji chrome 200 ASA colour film. The film was developed and printed commercially.

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